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March 2006

On the Cover

Rembrandt van Rijn (1606–1669).
Scholar in His Study (1634).
Oil on canvas (141 cm × 135 cm).
National Gallery in Prague, Czech Republic.

About the Cover p. 537

Perspectives

Possibilities for Relapsing Fever Reemergence 369
S.J. Cutler
Increasing globalization may pave the way for reemergence of relapsing fever.

Cost-effectiveness of West Nile Virus Vaccination 375
A. Zohrabian et al.
Universal vaccination is unlikely to result in societal monetary savings.

Synopses

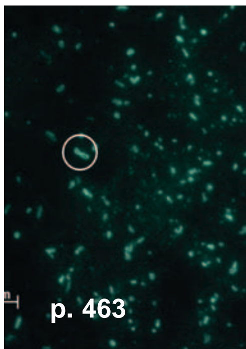
Web-based Surveillance and *Salmonella* Distribution 381
E. Galanis et al.
Surveillance improves control of *Salmonella* infections.

***Bartonella* in Pets and Human Health** 389
B.B. Chomel et al.
Pets represent a large reservoir for human infection.

Research

West Nile Virus Infections, United States, 2003 395
M.P. Busch et al.
Routine donor nucleic acid amplification testing is a valuable surveillance screening tool.

p. 370


p. 463

***Aspergillus ustus* Infections among Transplant Recipients** 403
A.A. Panackal et al.
This is the first report of clustered *A. ustus* causing systemic disease in transplant patients.

***Clostridium difficile* Infections, US Hospitals, 1996–2003** 409
L.C. McDonald et al.
Clinicians should be aware of the increasing risk for *C. difficile*–associated disease and make efforts to control its transmission.

Medication Sales and Syndromic Surveillance 416
E. Vergu et al.
Real-time over-the-counter drug sales provide an additional tool for disease surveillance.

Personal Hygiene and MRSA Infection .422
G. Turabelidze et al.
Improving personal hygiene practices may prevent and control MRSA outbreaks.

Rapid Detection of *Rickettsia prowazekii* 428
S. Svraka et al.
This highly standardized and adaptable assay could improve epidemic typhus surveillance.

Serosurvey on Household Contacts of Marburg Patients 433
M. Borchert et al.
Asymptomatic secondary infections are rare.

Canine Visceral Leishmaniasis, 2000–2003 440
Z.H. Duprey et al.
Foxhounds infected with *Leishmania* spp. were found in 18 states and 2 Canadian provinces.

Chemoprophylaxis and Malaria Death Rates 447
G. Krause et al.
Malaria chemoprophylaxis increases the survival of nonimmune travelers.

EMERGING INFECTIOUS DISEASES
March 2006

Self-medication with Antimicrobial Drugs in Europe 452
L. Grigoryan et al.
Antimicrobial drug self-medication occurs most often in eastern and southern Europe and least often in northern and western Europe.

Pneumonic Plague Cluster, Uganda, 2004 460
E.M. Begier et al.
In a case cluster, pneumonic plague transmission was compatible with respiratory droplet rather than aerosol transmission.

Mosquitoes and West Nile Virus Transmission 468
G. Molaei et al.
Culex salinarius is a bridge vector to humans, while *Cx. pipiens* and *Cx. restuans* are more efficient enzootic vectors.

Genotypes in Polyclonal Infections by Single Species 475
J.M. Colborn et al.
The combination of real-time PCR and capillary electrophoresis permits the rapid identification and quantification of pathogen genotypes.

Dispatches

483 **"*Candidatus Rickettsia kellyi*," India**
J.M. Rolain et al.

486 **Lyssavirus Surveillance in Bats, Bangladesh**
I.V. Kuzmin et al.

489 **Resistant *Salmonella* Paratyphi A Isolate, India**
S. Nair et al.

492 **Highly Pathogenic Canine Coronavirus**
C. Buonavoglia et al.

495 **Acute Hemorrhagic Conjunctivitis, Rio de Janeiro**
F.N. Tavares et al.

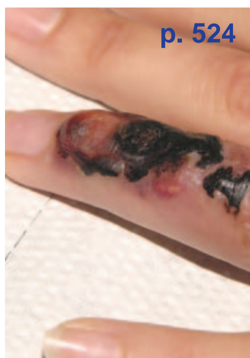
498 **Pneumonia and New MRSA Clone**
F. Garnier et al.

501 **Canine Leptospirosis**
G.E. Moore et al.

504 **Lagos Bat Virus, South Africa**
W. Markotter et al.

507 ***Salmonella* Paratyphi B in Aquariums**
R.S. Levings et al.

p. 487


p. 524

511 **Protease-resistant Prion Protein in Mice**
L. Cervenakova et al.

514 **West Nile Virus Paralysis Outcome**
J.J. Sejvar et al.

Letters

517 **Human Pythiosis**

518 **Rift Valley Fever Potential, Arabian Peninsula**

520 **Screening and Toxigenic Corynebacteria Spread**

521 ***Rickettsia slovaca* Infection, France**

523 **Cutaneous Anthrax, Belgian Traveler**

525 **Japanese Encephalitis, Singapore**

526 **HIV and Lacaziosis, Brazil**

527 **Hand Sanitizer Alert**

529 **Spleen Abscess as Malaria Complication**

531 **Rickettsioses in South Korea, Materials and Methods**

531 **Rickettsioses in South Korea, Data Analysis**

532 **"*Mycobacterium tilburgii*" Infections**

Book Reviews

535 **Infection and Autoimmunity**

535 **World Class Parasites: Vol. X, Schistosomiasis**

News & Notes

About the Cover
537 **Different Strokes for Different Folks in Search of Truth**

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Possibilities for Relapsing Fever Reemergence

Sally J. Cutler*

Relapsing fever *Borrelia* infections have attracted little attention in recent years; however, where endemic, these infections still result in considerable illness and death. Despite the marked antimicrobial drug susceptibility of these organisms, therapy is often delayed through lack of clinical suspicion. With increasing travel, infections may be imported, through exotic relapsing fever infection or through resurgence of infected disease vectors. Although louseborne relapsing fever is now geographically limited, it was once of global importance. The possibility for reemergence was recently highlighted by the probable reemergence of louseborne relapsing fever in homeless persons from France. Host limitations enforced through louseborne transmission are less applicable for the tickborne forms of relapsing fever. Although the latter have reduced potential for epidemic spread, they have the ability to infect diverse hosts, thus establishing reservoirs of infection and presenting greater challenges for their control.

Since the 1980s, the number of *Borrelia* species associated with relapsing fever has doubled. This situation is in part due to improved diagnostics and molecular techniques that have enabled sequenced-based characterization of these spirochetes. Seventeen species are attributed to the relapsing fever group of spirochetes; others have been described but await further characterization and isolation before species can be designated. Infection is vectorborne, primarily by *Ornithodoros* ticks, which led to the descriptive name of tickborne relapsing fever. However, 1 form of the disease developed epidemic potential by adapting to louse transmission and thus became known as louseborne relapsing fever.

Clinically, these spirochetes all produce an undulating febrile disease in humans, with signs and symptoms often indistinguishable from those of malaria. Diagnosis in most disease-endemic areas relies on demonstrating the spirochete in Giemsa-stained blood films; however, more discriminating methods are available that can be used in suitably equipped facilities.

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Whether we are likely to see the reemergence of this disease is difficult to predict because we do not understand relapsing fever borreliae and their complex host interactions. Indeed, to use the words of Bryceson et al., "Little is understood of where it lurks between epidemics and of how it suddenly springs up after silent intervals of several years" (*1*). The same authors describe the louseborne disease as the "most epidemic of the epidemic diseases." Certainly, increasing population movements and travel to disease-endemic areas are likely to introduce relapsing fever to areas where it is not been common. Here, the danger lies in not considering this diagnosis or mislabeling the disease as Lyme borreliosis because of the likely cross-reactive serologic results. Although the louseborne epidemic form of the disease was once distributed globally, it is now localized to a few countries. The recent documentation of probable cases in homeless populations in France raises the possibility of reintroduction of this disease in countries where it was believed to have been eliminated.

Discovery of the Disease

Compatible clinical disease descriptions have been documented since the time of Hippocrates; however, the term relapsing fever was first used by David Craigie to describe an outbreak of the disease in Edinburgh in 1843 (*1*). The spirochetal cause for louseborne relapsing fever was first demonstrated by Otto Obermeier during an outbreak in Berlin (1867–1868). His inability to reproduce the disease in animal models (and indeed in himself) delayed the publication of these findings until 1873 (*1*). The causative agent of the African tick variety of relapsing fever was discovered by Ross and Milne in 1904 (*2*). This finding was also made independently by Dutton and Todd, who demonstrated transmission by using infected *Ornithodoros moubata* ticks and a monkey model (*3*). The publication of their findings in 1905 resulted from the fact

that Dutton became infected with this spirochete. The role of the human body louse in the transmission of relapsing fever was reported by MacKie in 1907 (*1*).

Historical Epidemics and Endemic Disease Foci

During the first half of the 20th century, relapsing fever was a disease of major worldwide importance; it caused epidemics affecting ≈50 million and was associated with death rates of 10% to 40% (*1*). During the 1930s, approximately one third of the population in Africa was devastated by an epidemic attributed to relapsing fever. Since 1967, the epidemic form of louseborne relapsing fever has been largely confined to areas of extreme poverty in East Africa (*4*) and the Peruvian Andes; most cases occur in Ethiopia. A recent outbreak in neighboring Sudan is estimated to have affected 20,000 members of the Dinka tribe in 1998 and 1999; the death rate was 10%–14%. Despite the reappearance elsewhere in the world of other louseborne diseases, such as epidemic typhus in Burundi and trench fever in vagrant populations, little evidence of reemergence of louseborne relapsing fever exists. Furthermore, molecular analysis of lice collected from around the world, including France, Peru, Russia, and the African countries of Burundi, Congo and Zimbabwe, did not produce evidence of infection with the louseborne relapsing fever agent (*5*).

Tickborne relapsing fevers may be endemic or sporadic. They still cause major health problems in Africa; in areas such as central Tanzania, this disease is a substantial cause of child deaths (*4,6*). Although present in some European countries, Central Asia, the Middle East, and the Americas, tickborne relapsing fever tends to be rarer (*7–10*). It is often associated with camping out in rural locations in close proximity to animal reservoirs of the spirochete and their associated *Ornithodoros* tick vectors (Figure 1) (*7–9*).

Figure 1. *Ornithodoros moubata* ticks that frequent traditional homes.

Relapsing Fever Today

This disease still has persistent foci of infection, where control can be a major healthcare problem. Relapsing fever can be acquired by travelers and brought back to regions where the disease is not epidemic (*10,11*) after eco-challenges or in association with military training or activities such as camping or caving, provided susceptible hosts and natural disease ecologic cycles coincide (*8,9*). Although many would argue that this limited disease impact is not a threat to public health, the lack of consideration of relapsing fever as a potential cause of clinical findings is a cause for concern.

Tickborne Relapsing Fevers

Borrelia duttonii, the cause of tickborne relapsing fever, is endemic to several countries in East Africa, such as Tanzania. No accurate data on the number of cases in Tanzania exist because this infection is not reportable in the Ministry of Health's Health Management Information System. Estimates of the incidence are likely to be grossly underestimated because many cases are diagnosed as malarial treatment failures. Localized studies have shown that the annual incidence is 384/1,000 in children <1 year of age and 163/1,000 in children <5 years of age (*4*), while other researchers have reported an incidence of 59/1,000 (*12*). *B. duttonii* infection primarily occurs in children and pregnant women, and it is associated with fetal loss and neonatal deaths. A perinatal death ratio of 436/1,000 has been reported from disease-endemic regions of Tanzania (*4*).

The vector is the soft tick, genus *Ornithodoros*; the species complex *Ornithodoros moubata* is prevalent in sub-Saharan Africa. These ticks live in traditional housing and mainly feed nocturnally (Figures 2 and 3). The disease is transmitted either by saliva during tick feeding or in coxal fluid excreted during feeding. The tick feeds for a short time only (usually less than half an hour), then returns to the earth floor or walls of the house. Humans are

Figure 2. Traditional Tembe dwelling in Tanzania.

believed to be the only natural reservoir for *B. duttonii*, unlike the situation for *B. crocidurae* in West Africa, where rodents are reservoirs. (Up to 18% of rodents from Senegal are infected with this spirochete [*13*]). Human infection with *B. crocidurae* appears to be more prevalent than previously described, however (*14*). This increase could be attributed to improved diagnostic capabilities and sample processing (*15*), but it is largely due to molecular techniques that can facilitate not only detection but also subsequent identification through sequencing the target gene (*16,17*).

Reports of tickborne relapsing fever elsewhere appear to be more sporadic or involve limited clusters of infection, which typically are associated with events that bring susceptible human hosts into close proximity with the tick vectors and their usual wildlife reservoirs (*8,9*). The species most frequently incriminated in such cases include *B. turicatae* and *B. hermsii*. Isolated reports of *B. persica* infection transmitted by its *O. tholozani* vector have also appeared in the Middle East (*9*), and *B. hispanica* transmission has been reported in Spain and North Africa (*10*).

Louseborne Relapsing Fevers

Many now believe that louseborne relapsing fever can be assigned to the history books; however, considerable disease-endemic foci of infection remain in areas of Ethiopia, which spill into neighboring countries such as Sudan (*18*). This global reduction of louseborne relapsing fever has largely resulted from the demise of the human clothing louse through improved human living conditions. A cause for concern is the increasing preference for washing clothes at low temperatures, which could permit louse survival. Ideally, clothes should be washed in water >60°C to kill lice. These lice are unable to survive away from their human hosts for more than a matter of hours and consequently tend to persist in areas of extreme poverty or in association with major population upheavals and turmoil, such as wars or environmental disasters. The increase of clothing lice among less privileged persons in industrialized countries is a growing concern. Blood samples collected from homeless persons in France from 2000 to 2003 showed serologic reactivity against *B. recurrentis*, which suggests a small disease outbreak and serves as a reminder against complacency against controlling or eliminating this disease (*19*).

Potential for Introduction or Reintroduction into Industrialized Countries

The ability of these spirochetes to remain associated with their arthropod vectors throughout the lifespan of the vector is well established (*20*). However, most show limited capacity for transovarial transmission (*21*) since these spirochetes are typically transmitted through soft ticks or clothing lice, both of which only feed for a short duration (<30 minutes) before sequestering in cracks and crevices or seams of clothing.

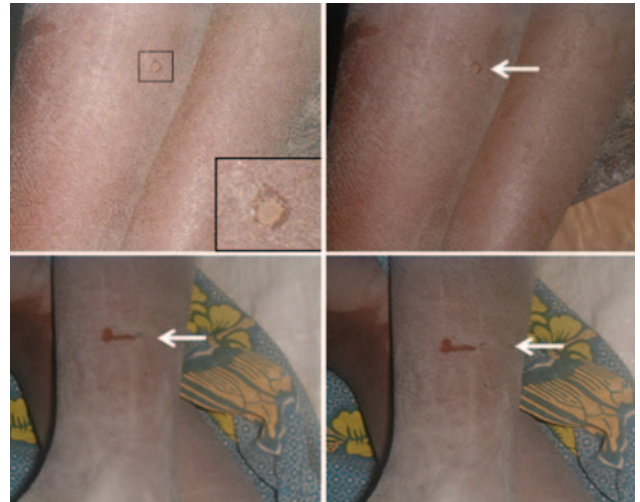

Figure 3. *Ornithodoros moubata* ticks feeding on sleeping children.

Neither soft ticks nor lice can "travel" from their particular ecologic niches. However, finding *Borrelia* spp. that greatly resemble relapsing fever borreliae with natural transmission through hard tick (ixodid) vectors means that these ticks may play a role in transmission. Ixodid ticks have been found in association with seafaring birds and songbirds, which raises the possibility of migration-associated transmission. Furthermore, the finding of novel *Borrelia* spp. in Africa that strongly resemble New World relapsing fever species suggests greater globalization of these spirochetes (*22–24*). As yet, the possible clinical importance of these spirochetes, for which the name "*Candidatus* Borrelia mvumii" has been proposed, remains unresolved (*16,22,23*). Studies are particularly hampered by the current lack of cultivatable strains.

Studies of rodents in California have shown a new potential borrelial species, tentatively named "*Borrelia davisii*," which appears distinct from both the Lyme-associated and relapsing fever groups of spirochetes. Despite the finding of this spirochete in blood samples collected from rodents, *Ixodes scapularis* ticks collected from the same geographic region were uniformly negative, which indicates that an alternative vector may exist for this spirochete. The clinical importance, if any, remains to be ascertained.

Lice are restrained by their exquisite host specificities. Human clothing lice have been adapted to feeding on a rabbit host (*20*); however, they still show strong preference for their human hosts. Whether other species of lice, or indeed other arthropod vectors, could transmit louseborne spirochetes is an open question. More recently, excretion

of *B. recurrentis* in louse feces has been demonstrated (*20*). Louse feces, in turn, could become aerosolized through desiccation and dispersal, potentially increasing possibilities for spread of this infection; however, this route of transmission currently is anecdotal.

The possibility that the agents of louseborne epidemic relapsing fever (*B. recurrentis*) and of East African tickborne relapsing fever (*B. duttonii*) may indeed be the same, or highly related clones of a common ancestor, has been raised (*24*). Phylogenetic comparisons have demonstrated the homology between these spirochetes and resemblance with the West African *B. crocidurae* species (*16,17,24*). More recently, analysis of an intragenic spacer typing method for these spirochetes, although showing clear differences between *B. crocidurae*, was unable to discriminate between *B. recurrentis* and *B. duttonii* (*24*). If indeed these strains are homogeneous, the potential transmission of the louseborne *B. recurrentis* by tick vectors must be considered.

Travel patterns have changed in recent years, particularly in more affluent nations; remote rural areas are particularly popular. Thus, imported cases associated with tourists returning to their home countries are likely (*10*). Vacation-associated infection with *B. hermsii* and *B. turicatae* has been reported from the Americas (*8*), while travel-associated cases of *B. crocidurae* and *B. hispanica* infection have been reported in Europe (*10,15*). Marked differences in the incidence of disease have been noted between civilian populations, who are becoming increasingly urbanized, and military populations, who have greater potential contact with rural environments during training exercises (*9*). Subsequent febrile illness is likely to be diagnosed as malaria; however, microscopic examination of Giemsa-stained blood films should show the spirochetal cause of such cases. Of concern is the ability of spirochetes to persist within the blood of patients long after fever resolves (*10*). Several mechanisms facilitate this persistence, including antigenic variation, binding of factor H, and rosetting of erythrocytes (*25–27*). Furthermore, increased movements of animals, their produce, and even fomites make alternative vehicles for transportation of these diseases likely, either through undiagnosed infection or transport of the vectors themselves. This was likely the route for introduction of West Nile virus into the United States.

Reservoirs of Infection and Vector Control

Natural Reservoirs

The role of wildlife as natural reservoirs for *Borrelia* spp. (particularly rodents, warthogs, or other mammalian hosts) is well established; most relapsing fevers rarely affect humans (*13,28*). In contrast, *B. duttonii* and *B. recurrentis* have no identified natural hosts other than humans. For these 2 spirochetes, whether the vector is also the reservoir (with the human's role being amplification of infected vectors) or if humans can be considered as the reservoir for infection is debatable. Recent work by Kisinza et al. in the Dodoma region of Tanzania found 5% of febrile children had positive blood slides for *Borrelia* spp. (*22*). Others studying nonfebrile persons have reported that 3% of them are spirochetemic by blood film examination. Furthermore, our own population studies on incidence in randomly chosen villagers in 4 Tanzanian villages showed that 11% were positive for borrelial DNA by using polymerase chain reaction. Whether this level of infection is sufficient to sustain the disease is not known.

Livestock

The role of livestock as hosts is not well established; however, a recent case report described equine abortion associated with a spirochete resembling *B. parkeri* and *B. turicatae* (*29*). This case also indicates that infection may be associated with livestock transportation. Whether other livestock or companion animals are susceptible to infection remains to be established.

Vectors

Many diseases now considered to be emerging or reemerging threats are vectorborne. Factors contributing to this phenomenon include changes to natural habitats, climatic change, and different levels of vector control strategies.

The stringent host specificity of the louse vector is the probable reason for the current demise of louseborne relapsing fever. In contrast, tick vectors are less host-specific; thus, spirochetes can be transmitted to more diverse hosts. Short-term vector control methods can be instigated, such as through use of acaricides. Although these methods have met with some success, we also need more affordable, yet sustainable, means of intervention that are likely to break the natural transmission cycles or reduce contact between reservoir, vector, and susceptible hosts (*6*). Improvements in housing design or institution of physical barriers such as bed nets could reduce vectors' access to susceptible hosts. Although these measures could reduce disease under certain circumstances, they offer little protection for populations at high risk, such as the homeless in industrialized countries.

Host-Pathogen Relationships

Spirochetes must be maintained within the circulatory system of the host, thus enabling transmission to uninfected co-feeding or subsequently feeding arthropod vectors. Host blood persistence appears to be the result of many interactions between the host and spirochete. The ability of

these spirochetes to undergo antigenic variation is well documented (*25*). Briefly, successive waves of spirochetemia characteristically seen in relapsing fever are associated with different antigenic variants, which result from rearrangement of silent variable membrane protein genes into an expression locus. This repertoire may be further extended through the multiplicity of mosaiclike variable membrane protein genes and pseudogenes possessed by some members of this group. These genes may have a role in extending antigenic diversity akin to the combinational gene conversion described in *Anaplasma marginale* (*30*).

This description does not address the question of whether spirochetes associated with clinical cases differ from those that do not appear to cause overt clinical signs. Immunity is a factor, highlighted by the high prevalence of disease in young children and pregnant women. However, for humans to serve as reservoirs for this disease, the spirochete must persist in sufficient numbers in a site where they will be acquired by new arthropod vectors. An intriguing hypothesis is that particular antigenic variants of the spirochete may be associated with life in an arthropod vector (usually ticks, except for the related spirochete, *B. recurrentis*), while others are suited to persistence in their human host (disease reservoir); additional types are associated with patients with overt clinical signs mediated through a multiplicity of host-microbe interactions. Persistence may be further facilitated through the neurotrophic tendency of these spirochetes. This hypothesis is further strengthened by observed pathologic differences associated with isogenic variants of the same isolate of *B. turicatae* investigated in vivo (*31*).

Clinical manifestations and patient management fall largely outside the scope of this review. However, clinical findings are reported elsewhere (*1*), in particular, details of management of neurologic, respiratory, and cardiac complications of these diseases.

Both innate and acquired immune responses are needed to control infection. To overcome innate immune mechanisms, these spirochetes bind the complement inhibitor factor H to their surface, thus avoiding deposition of the membrane attack complex and subsequent lysis (*26*; Meri et al., unpub. data). Clearance of spirochetes is primarily mediated through production of specific bactericidal immunoglobulin M antibodies (*32*); complement is not required (*33*). This specific immunoglobulin is primarily directed against the outer membrane lipoproteins of these spirochetes. T cells do not appear to have a major role in the resolution of spirochetemia (*32*).

Another mechanism that may enhance persistence of these spirochetes within the bloodstream is the ability of some relapsing fever spirochetes to become coated with or to rosette blood erythrocytes. This process enables prolonged persistence through a masking or steric hindrance effect, which prevents interaction with the host immune cells (*27,34*). This ability to bind erythrocytes might be associated with pathologic observations, including vascular tissue damage, hemorrhages, and inflammation (*27*); however, this hypothesis would not explain similar pathologic observations in nonrosetting phylogenetically related borreliae.

Future Prospects

Traditionally, diagnosis is based on demonstration of spirochetes in blood films taken during the acute febrile period. More recently, an enzyme-linked immunosorbent assay that used the *GlpQ* gene product was demonstrated to be a useful diagnostic aid (*35*). Furthermore, this antigen is specific for the relapsing fever group borreliae, thus distinguishing these from cases of Lyme borreliosis (*35*).

Characterization of relapsing fever *Borrelia* spp. can now be facilitated through molecular approaches that allow more accurate investigation of which species are prevalent in different epidemiologic foci. Previously, such characterization relied on identification of disease vectors and compatibility with established geographic patterns. Furthermore, these techniques will enable characterization of newly discovered spirochetes (*36*) and identification of their reservoirs of infection. For example, spirochetes that were described in the guts of termites have now been allocated to a separate genus (*37*). Additional spirochetes have been identified in soil; however, their taxonomic status and clinical importance remain to be established (*38*). Other spirochetes have been discovered in various tick species, some showing rapid in vitro growth (*39*) and others producing no cultivable isolates to date (*22*).

Genome sequencing is under way for several relapsing fever spirochetes. This information will enable a thorough comparative analysis of these spirochetes and likely yield insights into vector competence and pathogenicity.

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Cost-effectiveness of West Nile Virus Vaccination

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West Nile virus (WNV) was first detected in the Western Hemisphere in 1999 in New York City. From 1999 through 2004, >16,600 cases of WNV-related illnesses were reported in the United States, of which >7,000 were neuroinvasive disease and >600 were fatal. Several approaches are under way to develop a human vaccine. Through simulations and sensitivity analysis that incorporated uncertainties regarding future transmission patterns of WNV and costs of health outcomes, we estimated that the range of values for the cost per case of WNV illness prevented by vaccination was US $20,000–$59,000 (mean $36,000). Cost-effectiveness was most sensitive to changes in the risk for infection, probability of symptomatic illness, and vaccination cost. Analysis indicated that universal vaccination against WNV disease would be unlikely to result in societal monetary savings unless disease incidence increases substantially over what has been seen in the past 6 years.

West Nile virus (WNV) was first detected in the Western Hemisphere in 1999 during an outbreak of encephalitis in New York City (*1*). Over the next 6 years the virus spread across the continental United States, as well as into Canada, Latin America, and the Caribbean islands (*2,3*). From 1999 through 2004, >16,600 WNV illnesses in humans have been reported in the United States; >7,000 of these were neuroinvasive disease, and >600 were fatal. In 2002 alone, 2,942 cases of neuroinvasive WNV disease were reported in the United States, which represents the largest epidemic of neuroinvasive WNV disease ever recorded (*4*). Approximately 20% of WNV infections in humans result in symptomatic illness, and ≈1% of infections lead to encephalitis, meningitis, or acute flaccid paralysis (*1*). A substantial proportion of persons in whom severe neuroinvasive WNV disease develops have long-term disability or die as a result of their infection (*5,6*).

WNV is transmitted to humans primarily through the bite of infected mosquitoes, but transmission through blood transfusion, through organ donation, and from mother to child have been described (*7*). Strategies to prevent WNV infection include avoiding exposure to infected mosquitoes, reducing the abundance of mosquito vectors, and screening infected blood donations before transfusion. Several approaches are under way to develop a safe and effective human vaccine (*8–10*). The public health utility of a new vaccine will depend largely on the incidence, geographic distribution, and severity of WNV disease in the United States, as well as the cost of vaccination. We evaluated the cost-effectiveness of vaccination against WNV in the United States from a societal perspective. Uncertainties regarding the future transmission patterns of WNV and the costs of health outcomes preclude an exact estimation of the economic impact of vaccination. Through probabilistic sensitivity analysis, which incorporates these uncertainties, we estimated the range of most likely values for the cost-effectiveness of vaccination and described the variables that have the most impact on the economic outcome of vaccination. We also estimated the likelihood that a universal vaccination program would result in economic savings.

Methods

The decision tree used to evaluate the cost-effectiveness of vaccination compared with no vaccination is shown in the Figure. Vaccination was assumed to have no effect on the incidence of infection or the severity of WNV illness but rather to influence only the proportion of infected persons in whom symptoms would develop. Baseline probabilities for each of the chance nodes in the tree were derived by reviewing published articles on the incidence, clinical manifestations, and outcomes of WNV disease as described in further detail below. We estimated the average cost per case of WNV illness prevented, that is, average cost-effectiveness ratio (ACER) (online Appendix 1, available from http://www.cdc.gov/ncidod/EID/vol12no03/05-0782_app1.htm) by calculating the expected societal costs of WNV illness with a vaccination strategy, subtracting the costs of illness with no vaccination, and dividing the

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Figure. Decision tree for vaccination program. WNV, West Nile virus.

remainder by the number of cases prevented by vaccination.

Time Horizon

We assumed that a single dose of live-attenuated WNV vaccine would provide immunity for ≥10 years, as is true for the currently licensed yellow fever vaccine (*10,11*). If an inactivated vaccine were used, 2 or 3 initial doses would probably be required, and booster doses would probably be needed every 3 years, as is currently recommended for inactivated Japanese encephalitis vaccine (*12*). Both the cost and effectiveness of vaccination were assumed to be the same whether achieved through a single live-attenuated vaccine dose or multiple inactivated vaccine doses at a lower cost per dose.

Although the time horizon for risk for illness, protection from the vaccine, and cost of vaccination was 10 years, we used estimated lifetime costs of disease outcomes in our model. Thus, we modeled the difference in lifetime costs of illness that would be incurred by society during a 10-year period under an immediately implemented universal vaccination strategy compared with no vaccination.

The probabilities of outcomes and costs modeled are average probabilities for the entire population, regardless of age. Our analysis therefore estimates ranges of average societal costs and outcomes prevented when all people in the society are vaccinated, regardless of the age at vaccination or illness. A more detailed analysis of the effect of vaccinating certain age groups would require estimates of age-specific risks and costs of outcomes, which are not readily available for most outcomes in the model.

Estimation of Costs

The overall cost of WNV illness per person at risk was calculated as the sum of the average costs for each health outcome weighted by the probability of occurrence of each outcome (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no03/05-0782.htm#table_app). Both medical treatment costs and productivity losses due to illness and death from WNV infection were included in cost estimates. We considered the following health outcomes of WNV infection in our analysis: asymptomatic infection, uncomplicated febrile illness with full recovery, neuroinvasive illness (encephalitis, meningitis, or paralysis) with full recovery, neuroinvasive illness with residual long-term disability, and death.

Asymptomatic infection was assumed to have no cost. Estimates of the cost of uncomplicated febrile illness due to WNV infection were not available so we assumed a cost of US $1,000 per case, based on 5 days of lost productivity at $165 per day (*13*), plus an assumed $175 in medical costs that included 1 ambulatory care visit, diagnostic tests, and outpatient medications. Precision of this cost estimate was not very important since the cost-effectiveness ratio was not sensitive to the changes in this variable. The estimated cost per case of neuroinvasive WNV illness with full recovery ($27,500) was derived from an economic study conducted during the 2002 WNV epidemic in Louisiana (*14*) in which economic costs, rather than charges, were considered a measure of resources. Our goal was to measure the forgone benefits that could have been derived if the resources had been allocated to their next best use, i.e., the opportunity cost. Charges made by healthcare providers do not usually reflect the opportunity costs because of healthcare market imperfections. Charges were adjusted to economic costs through the use of cost-to-charge ratios (for details see Appendix 2 in reference *14*; we adjusted 2002 dollars to 2004 dollars, the last year for which consumer price indices were available at the time of this study [*15,16*]). This cost of neuroinvasive illness included costs of outpatient evaluation, inpatient treatment, rehabilitation treatment, lost productivity of the patient and caregiver at home, and transportation (online Appendix Table). Estimates of the cost of residual long-term disability after neuroinvasive disease were not available, but many of the disabilities that have been described after WNV illness are clinically similar to those that result from acute stroke, and the 2 conditions both affect primarily older males. We therefore used estimates of the lifetime cost of stroke as a proxy for the cost per case of neuroinvasive WNV illness with residual long-term disability (*17*) (1990 dollars adjusted to 2004 dollars [*15,16*]). Details are shown in online Appendix 2 (available from http://www.cdc.gov/ncidod/EID/vol12no03/05-0782_app2.htm).

The average societal cost due to death from WNV disease was estimated by using productivity loss tables (*13*) and the age distribution of 713 WNV nationwide deaths reported to the ArboNET database of the Centers for Disease Control and Prevention (CDC) since 1999 (CDC, unpub. data). The median age of fatal cases was 77 years (range 1 month to 99 years). The estimated cost due to death was $200,000 at a 3% discount rate (2000 dollars from productivity tables [*13*] were adjusted to 2004 dollars [*16*]). Since short-term costs in our model were randomly distributed throughout the 10-year time horizon, to simplify the model, we only discounted the long-term costs, such as long-term disability costs and costs due to death. For the short-term costs incurred within the 10-year time horizon, we assumed our estimates represented the present values of those costs (online Appendix 2, available from http://www.cdc.gov/ncidod/EID/Vol12No03/05-0782_ app2.htm).

Since no human WNV vaccine was licensed at the time of our evaluation, vaccine costs were not available. Based on charges in the United States for yellow fever vaccine (≈$85 per dose), hepatitis A vaccine (≈$75 per dose), Japanese encephalitis vaccine (≈$315 for a 3-dose series), and the previously available Lyme disease vaccine (≈$150 for a 3-dose series), we assumed a total baseline vaccination cost of $100 to include both the actual cost of the vaccine and the cost of administering the vaccine. For the sensitivity analysis focused on the cost of vaccination, we assumed minimum and maximum vaccination costs of $10 and $150, respectively (see below).

Estimates of Probabilities for Health Outcomes

Probability of Infection

Several seroepidemiologic surveys have estimated the proportion of North American populations who were infected with WNV during epidemic transmission. The highest seroprevalence published to date is 2.6% (*1*). In 2002, during the largest epidemic of WNV neuroinvasive disease ever described in the United States, 2,942 neuroinvasive WNV disease cases were reported from 36 states and the District of Columbia (total population ≈253.4 million). If one assumes a ratio of 1 neuroinvasive case for every 140 infections, which was the finding of a 1999 household-based seroepidemiologic survey in New York City (*1*), this yields an overall estimate of ≈411,880 infections and an estimated incidence of 0.16 infections per 100 people, or 0.0016 per person per year. Whether WNV epidemics will continue to occur in the United States at a similar frequency or intensity is unknown, but for this analysis we assumed that the risk for WNV infection would be 0.0016 per person per year for 10 years. The cumulative risk for WNV infection over a 10-year period would be $1 - e^{(-0.0016 \times 10)} = 0.016$. We therefore estimated the baseline probability of infection as 0.016. For sensitivity analysis focused on probability of infection, we assumed for the minimum risk for infection that a person would encounter only 1 year of WNV transmission, yielding a cumulative risk of 0.0016 over the 10-year period. For the maximum risk, we assumed that the risk would be that of yearly epidemic transmission such that 2.6% of the population would be infected each year over the 10-year period, yielding a 10-year cumulative risk of 0.23. Further details regarding sensitivity analysis are described below.

Probability of Symptomatic Illness and Vaccine Effectiveness

We assumed that symptoms of WNV illness will develop in 20% of infected persons and that neuroinvasive disease will develop in 3.6% of them, which is equivalent to 1 neuroinvasive case for every 140 infections previously described (*1*). We also assumed a vaccine effectiveness of 80% in reducing the risk for symptomatic illness.

Probability of Long-term Disability or Death after Neuroinvasive WNV Disease

Precise data on long-term outcomes from WNV illness are limited. A study of 19 patients with neuroinvasive WNV disease found that 2 (11%) died, and of the 17 survivors, 7 (41%) had recovered fully at the time of discharge, 6 (31%) were discharged without full recovery, and 4 (24%) were discharged to a long-term care facility (*18*). Another study of 57 patients with neuroinvasive disease found that 10 (18%) eventually died, 13 (23%) were discharged without support, 14 (25%) were discharged requiring support, 14 (25%) were discharged to a rehabilitation facility or nursing home, 4 (7%) moved in with relatives, and 2 (4%) remained in an acute care facility (*5*). A study of 16 patients with neuroinvasive WNV disease found that 1 patient (6%) died and that 8 months after illness, 4 (25%) patients required assistance or rehabilitation and 11 (69%) were functioning independently at home (*19*). A survey of 35 patients who had been hospitalized with WNV illness found that 63% reported full recovery 12 months after illness onset (*6*). Based on the limited data from these studies, we assumed that 35% of patients would have lifelong disability after neuroinvasive WNV disease. Of 2,942 patients with neuroinvasive WNV disease reported in the United States in 2002, 276 (9%) died (*4*). For our model, we assumed a case-fatality ratio of 9%.

Sensitivity Analysis

To incorporate uncertainties regarding the values of all input variables, we assigned uniform probability distribution to all variables, allowing 25% variability around the baseline values (Table 1). We used @Risk Analysis 2002 software (Palisade Corporation, Newfield, NY, USA) to

generate distributions of possible outcomes by Monte Carlo simulation of the ACER using 5,000 iterations that covered all combinations of input variable values. The results provided detailed summary statistics for the ACER distribution, including the 5th and 95th percentile ranges of values and the probability that vaccination would result in societal savings. To further investigate the impact of the risk for infection and vaccination cost on the ACER, we ran separate simulations in which the minimum, baseline, and maximum values for these variables described in the corresponding sections were fixed, while all other variables were allowed to vary according to their prespecified uniform distributions.

Results

Using baseline values of all input variables, without accounting for uncertainties, the average cost per case of WNV illness prevented would be ≈$34,200. At a cost of $8.7 billion in a hypothetical population of 100 million people, vaccination would prevent 256,000 cases of WNV illness, including 9,216 cases of neuroinvasive disease, 2,935 cases of lifetime disability, and 829 deaths during a 10-year period. Under these assumptions, universal vaccination would yield societal savings if the cumulative incidence of WNV infection over a 10-year period were ≥0.13 (≈1.4% of the population infected each year), the cost of vaccination were ≤$12.8, or the cost of lifelong disability were ≥$3.2 million (≈15 times higher than the baseline estimate).

The simulation results accounting for uncertainties in all input variables are shown in Table 2. The median of the ACER distribution was $35,000 per case of WNV illness prevented. The 5th and 95th percentiles for the ACER were $59,000 and $20,000, respectively.

To identify the sensitivity of the output to all input distributions, we used @Risk sensitivity analysis with a regression in which the dependent variable was the output variable, i.e., ACER, and the independent variables were the input variables presented as @Risk uniform distribution functions (Table 1). Each iteration represented an observation for the regression. The coefficients calculated for each input variable measured the sensitivity of the output to that particular input distribution. The results indicated that ACER was most sensitive to the changes in the risk for infection, probability of symptomatic illness, and vaccination cost (Table 3). A 1 standard deviation (SD) increase in the probability of symptomatic illness increased the ACER by an SD of 0.65, while a 1 SD increase in the probability of infection or the vaccination cost increased the ACER by an SD of 0.5. Changes in the other variables had little or no impact on ACER (Table 3).

The results of the sensitivity analysis focused separately on risk for infection and vaccination cost are shown in Table 4. The probability that vaccination would yield societal savings changed from 0% to 98% when the 10-year cumulative risk for WNV infection changed from 0.016 to 0.230, and from 0% to 76% as the vaccination cost decreased from $150 to $10.

Discussion

The economic impact of a vaccination strategy is a determinant of the public health decision regarding whether or not to recommend vaccination, but it is certainly not the only determinant of sound public health vaccination policy. It is also not imperative that a vaccination program result in monetary savings for it to be cost-effective compared with other public health interventions. Societies and people are willing to pay for preventing disease, as indicated by the implementation of preventive interventions that do not result in economic savings, and most relevant, the willingness to pay for expensive vaccines (*20–22*). However, as public health implications of vaccination programs are considered, we must have some understanding of the resources that might be expended. Vaccination would be most appealing if it is likely to safely prevent disease and save society money, or at least have a relatively low cost per case of illness prevented.

Table 1. Uniform distributions for each variable used in simulations to assess the cost-effectiveness of vaccination against West Nile virus (WNV)*

Variable	Lower limit	Baseline	Upper limit
Probability of infection	0.012	0.016	0.02
Probability of symptomatic illness	0.15	0.20	0.25
Probability of symptomatic illness after vaccination†	0.03	0.04	0.05
Probability of neuroinvasive disease, given symptoms	0.027	0.036	0.045
Probability of death, given neuroinvasive disease	0.07	0.09	0.11
Probability of disability, given neuroinvasive disease	0.26	0.35	0.44
Cost of neuroinvasive disease	$20,625	$27,500	$34,375
Cost of death (direct and indirect financial losses)	$150,000	$200,000	$250,000
Cost of lifelong disability	$158,000	$210,000	$263,000
Cost of uncomplicated WNV febrile illness	$750	$1,000	$1,250
Cost of vaccination	$75	$100	$125

*Upper and lower limits are calculated as ±25% of the baseline values and rounded up.
†Baseline vaccine effectiveness is assumed to be 80%.

Table 2. Outcome distributions of average cost-effectiveness ratio (ACER) accounting for variability in all input variables*

Statistic	ACER†
5th–95th percentile range, $	–59,000 to –20,000
Mean, $	–36,000
Median, $	–35,000
Mode, $	–33,000
Probability of savings, %	0

*According to the distribution provided in Table 1.
†Negative value indicates cost.

Our analysis indicates that a universal vaccination program to prevent WNV disease would be unlikely to result in societal monetary savings unless the incidence of the disease increases substantially over what has been seen in the past 6 years, or the cost of vaccination were <$12 per person vaccinated. The risk for WNV infection, probability of symptomatic illness after infection, and cost of vaccine appeared to have the greatest influence on the cost-effectiveness outcome. Within the range of possible values used in our model, variations in vaccine effectiveness, cost of WNV illness, and probabilities of various health outcomes did not lead to considerable change in the cost-effectiveness.

The future patterns of WNV transmission in North America cannot be accurately predicted. The virus was first detected in North America in 1999, and the epidemiology of WNV illness in the Western Hemisphere continues to evolve. The antigenically related flaviviruses St. Louis encephalitis virus (SLEV) and Japanese encephalitis virus (JEV) demonstrate different patterns of transmission that WNV could assume; SLEV is sporadically transmitted in North America with intense epidemics separated by years of low-level transmission, while JEV occurs in Asia with annual epidemics of intense transmission. If WNV assumes a transmission pattern in North America similar to that of JEV in Asia, then vaccination is likely to be a much more appealing public health prevention strategy and is likely to be more cost-effective than if WNV transmission follows the pattern of SLEV. As WNV spreads southward into Latin America, increased incidence may be seen with less protection from mosquitoes provided by air conditioning and screens (*23*). If intense transmission is seen in these areas, vaccination may be the most cost-effective prevention strategy, but unless the vaccine cost is low, it may still be too expensive for local economies.

WNV infection may cause severe untreatable neurologic disease. While the risk is highest in the elderly, severe disease does occur among young adults and children (*4,24*). The more severe, untreatable manifestations of WNV infection would compel interest in vaccine development and use even if vaccination is expensive, particularly since current prevention strategies such as personal repellent use or environmental reduction of mosquito abundance may not be consistently implemented (*25*). The effectiveness of these other prevention strategies is difficult to conclusively demonstrate and estimates of their cost-effectiveness have not been published. Vaccination may reduce the expenditures for mosquito control in certain areas, but we did not include this possible effect in our model. If alternate prevention costs were reduced by vaccination, we would expect this to improve the cost-effectiveness of vaccination from the societal perspective.

Our results provide a general assessment of the likely economic implications of universal vaccination against WNV and an indication of which parameters have the greatest influence on the cost-effectiveness of vaccination. A safe and effective vaccine may prove to be the most effective, and perhaps the most cost-effective, strategy to prevent severe WNV illness. The economic impact of vaccination will depend mostly on the risk for WNV infection, probability of symptomatic illness after infection, and the cost of vaccination.

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Table 3. Sensitivity of the average cost-effectiveness ratio (ACER) for input variables

Rank	Input variables	Regression coefficient†
1	Probability of symptomatic illness	0.65
2	Probability of infection	0.51
3	Vaccination cost	0.50
4	Probability of symptomatic illness after vaccination	–0.14
5	Probability of neuroinvasive disease, given symptoms	0.05
6	Cost of lifelong disability	–0.03
7	Probability of disability, given neuroinvasive disease	0.03
8	Cost of neuroinvasive disease	–0.02
9	Cost of uncomplicated WNV febrile illness*	–0.01
10	Cost of death (direct and indirect financial losses)	–0.01
11	Probability of death, given neuroinvasive disease	0.00

*WNV, West Nile virus.
†@Risk analysis software runs a regression where the dependent variable is the output variable, i.e., ACER, and the independent variables are the input variables presented as @Risk uniform distribution functions. Each iteration represents an observation for the regression. The coefficient calculated for each input variable measures the sensitivity of the output to that particular input distribution. For example, a coefficient of 0.65 indicates that a 1–standard deviation (SD) increase in probability of symptomatic illness increases the ACER by an SD of 0.65.

Table 4. Sensitivity of the average cost-effectiveness ratio for stepwise changes in infection rate and vaccination cost*

	Infection rate			Vaccination cost		
Statistic	0.0016	0.016	0.23	–150	–100	–10
5th–95th percentile range, $	–585,000 to –261,000	–54,000 to –22,000	343 to 3,846	–86,000 to –36,000	–56,000 to –23,000	–1,400 to 2,900
Mean, $	–400,000	–36,000	2,096	–57,000	–36,000	860
Median, $	–386,000	–34,000	2,098	–55,000	–35,000	920
Mode, $	–373,000	–30,000	1,500	–54,000	–36,000	740
Probability of savings, %	0	0	98	0	0	76

*All other variables were allowed to vary according to their specified uniform distributions provided in Table 1. Negative value indicates cost; positive value indicates monetary saving.

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Web-based Surveillance and Global *Salmonella* Distribution, 2000–2002

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Salmonellae are a common cause of foodborne disease worldwide. The World Health Organization (WHO) supports international foodborne disease surveillance through WHO Global Salm-Surv and other activities. WHO Global Salm-Surv members annually report the 15 most frequently isolated *Salmonella* serotypes to a Web-based country databank. We describe the global distribution of reported *Salmonella* serotypes from human and nonhuman sources from 2000 to 2002. Among human isolates, *Salmonella enterica* serovar Enteritidis was the most common serotype, accounting for 65% of all isolates. Among nonhuman isolates, although no serotype predominated, *S.* Typhimurium was reported most frequently. Several serotypes were reported from only 1 region of the world. The WHO Global Salm-Surv country databank is a valuable public health resource; it is a publicly accessible, Web-based tool that can be used by health professionals to explore hypotheses related to the sources and distribution of salmonellae worldwide.

Foodborne diseases are among the most serious health problems affecting public health and development worldwide (*1*). Industrialization, mass food production, decreasing trade barriers, and human migration have disseminated and increased the incidence and severity of foodborne diseases worldwide (*2–4*).

Salmonellae are among the most common bacterial foodborne pathogens worldwide (*4*). They cause an estimated 1.4 million cases of foodborne disease each year in the United States alone (*5*). *Salmonella* serotyping is a surveillance tool that detects widespread outbreaks, identifies outbreak sources, monitors trends over time, and attributes human disease to various foods and animals (*6*). Such surveillance is needed to help prevent foodborne disease outbreaks and raise awareness among health authorities, food producers, food regulators, and consumers (*7*).

A 1997 survey of national reference laboratories showed that only 69 (66%) of 104 responding countries conducted routine *Salmonella* serotyping for public health surveillance (*8*). Consequently, the World Health Organization (WHO), the US Centers for Disease Control and Prevention, and the Danish Veterinary Laboratory (now the Danish Institute for Food and Veterinary Research) founded WHO Global Salm-Surv in 2000. Its mission is to promote integrated, laboratory-based surveillance and foster collaboration among human health, veterinary, and food-related disciplines to enhance the capacity to detect, respond, and prevent foodborne diseases (*9*). By November 2005, WHO Global Salm-Surv had >800 members from 142 countries. A key component of this program is the Web-based country databank, to which member countries annually submit their 15 most frequently isolated *Salmonella* serotypes. This program is the only foodborne disease surveillance network that is global in scope and surveys all aspects of the food chain, from animal feed to humans. Data are updated annually and are publicly accessible for members and the scientific community to review (www.who.int/salmsurv). We describe the global distribution of *Salmonella* serotypes from human and nonhuman sources reported to the WHO Global Salm-Surv country databank from 2000 to 2002 and explore how the databank may become a valuable public health resource for foodborne disease surveillance.

Methods

WHO Global Salm-Surv has conducted annual regional training courses for national reference laboratories since

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1999 and has managed an external laboratory quality assurance program since 2000 to facilitate a standard approach to isolating and serotyping salmonellae (*10*). National reference laboratories can become WHO Global Salm-Surv members and share *Salmonella* serotype data with other members through the country databank. The country databank is a Web-enabled Oracle database that is password protected for data entry and accessible for public viewing at www.who.int/salmsurv. Each year, a designated national reference laboratory representative enters into the country databank the number of *Salmonella* isolates serotyped from human, animal, food, environmental, and feed sources and the 15 most frequently identified serotypes.

Descriptive analysis was conducted by using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) on data from all countries that submitted data for 2000, 2001, or 2002 as of June 2004. Analyses for trends over time were conducted on data from 2000 to 2002. More detailed analyses, including ranking of serotypes, comparison of human to nonhuman isolates, and regional comparisons are presented for 2002 data only, the year in which the most countries participated.

Before 2001, nonhuman isolates were grouped together. Since 2001, countries have been able to submit food, animal, environmental, and feed data separately. For comparison purposes, all nonhuman data were combined in this analysis.

Data were grouped into regions approximately corresponding to 6 geopolitical continents: Africa, Asia, Latin America and the Caribbean, Europe, North America, and Oceania. To accommodate local epidemiologic characteristics, New Caledonia was incorporated into Asia, and Israel was incorporated into Europe. For years in which a single country contributed data for a region, regional data are not presented, but the data are included in the overall results. A region-specific serotype was defined as a serotype that, for each of the years of the study period, was among the 15 most commonly reported serotypes and for which >90% of the isolates were from that region.

Results

Global

Forty-nine countries submitted data to the WHO Global Salm-Surv country databank from 2000 to 2002 (Table 1). Twenty countries reported both human and nonhuman results, 21 reported only human results, and 8 reported only nonhuman results. Reports of 376,856 human and 65,789 nonhuman *Salmonella* isolations were entered into the database during the 3-year period. North America and Europe accounted for 87.9% (389,134) of all reported isolates. The number of isolates reported to the country databank was stable during the study period; 113,782–137,329 human isolates and 16,506–25,761 nonhuman isolates were reported per year.

During the 3-year period, *Salmonella enterica* serovar Enteritidis was by far the most common serotype reported from human isolates globally. In 2002, it accounted for 65% of all isolates, followed by *S.* Typhimurium at 12% and *S.* Newport at 4%. Among nonhuman isolates, *S.* Typhimurium was the most commonly reported serotype in all 3 years, accounting for 17% of isolates in 2002. It was followed by *S.* Heidelberg (11%) and *S.* Enteritidis (9%) (Figure 1).

In 2002, 26 (84%) of the 31 countries that reported human serotype results ranked *S.* Enteritidis and *S.* Typhimurium in their 10 most common human serotypes (Table 2). Approximately half of the countries ranked *S.* Infantis and *S.* Typhi in their 10 most common serotypes, but only a fourth ranked *S.* Newport and *S.* Heidelberg in their top 10. The relative ranking of serotypes by the number of countries reporting them in their 15 most frequent serotypes remained stable over the study period (data not shown). However, the proportion of countries reporting each serotype varied. For example, from 2000 to 2001, more than two thirds of countries reported *S.* Agona, compared to 39% in 2002.

In 2002, a total of 5 serotypes were reported among the 15 most common human serotypes from all 6 regions of the world: *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Montevideo, and *S.* Typhi. However, the proportion of isolates of each serotype varied greatly. In 2002, for example, *S.* Enteritidis represented 85% of isolates in Europe but only 9% in Oceania. In Latin America and the Caribbean, *S.* Typhi accounted for the greatest proportion of salmonellae (13%). In 2000 and 2001, *S.* Enteritidis, *S.* Typhimurium, *S.* Typhi, and *S.* Agona were reported from all 6 regions (data not shown).

S. Enteritidis, *S.* Typhimurium, and *S.* Typhi were ranked among the 15 most common human serotypes in all 6 regions throughout the 3-year study period. *S.* Agona, *S.* Infantis, *S.* Montevideo, *S.* Saintpaul, *S.* Hadar, *S.* Mbandaka, *S.* Newport, *S.* Thompson, *S.* Heidelberg, and *S.* Virchow were also widespread; they were reported from 4 to 6 of the regions from 2000 through 2002. Reporting of *S.* Montevideo increased from 4 regions in 2000 to all 6 regions in 2002. The reporting of *S.* Heidelberg increased from 3 to 5 regions in the same timeframe.

Regional

In Africa in 2002, *S.* Enteritidis and *S.* Typhimurium were each reported from approximately one fourth of isolates from humans (Figure 2). Among nonhuman sources (Figure 3), *S.* Anatum and *S.* Enteritidis constituted the largest proportion of isolates.

Table 1. Number of serotyped *Salmonella* isolates reported to the World Health Organization Global Salm-Surv country databank, 2000–2002

	Human			Nonhuman		
Country	2000	2001	2002	2000	2001	2002
Africa	104	406	965	33	101	1,477
Cameroon		263	247		12	10
Mali			34			
Morocco			76			
Senegal	104	143	220	33	89	91
Tunisia			388			1,376
Asia	8,233	6,696	5,771	4,056	1,513	1,631
China				43	98	127
Indonesia				219		
Japan	2,631	2,452	1,890			
Korea	1,260	918	843			
Malaysia	499			1,390		
New Caledonia		30	20			
Philippines	606					
Thailand	3,233	3,279	2,922	2,404	1,415	1,504
Vietnam	4	17	96			
Europe	91,788	73,556	85,385	10,628	8,951	3,113
Belgium	13,642	10,260				
Bulgaria	789	1,001	1,482			
Cyprus				52		45
Czech Republic	4,774	4,030	27,381			
Denmark	2,063	2,632	1,844	5,981	5,402	
Estonia				178	38	78
Germany				3,068		
Greece				337		842
Hungary	16,271	14,462	14,678	748	1,448	1,809
Israel	4,428	4,043	3,859			
Latvia					139	132
Luxembourg	381					
Norway	1,289	1,639				
Poland	38,138	26,601	28,705	234	524	151
Portugal	354	539				
Serbia and Montenegro	5,172	5,003	4,873		46	
Slovakia				30	1,354	56
Slovenia	3,456	1,576	2,563			
Switzerland	1,031	1,770				
Latin America and Caribbean	2,054	2,239	2,491	411	633	727
Argentina	633	608	487	124	165	147
Barbados		27	71		24	23
Bolivia		19	9		8	2
Chile	929	920	1,284	218	329	395
Colombia	145	135	194		31	52
Costa Rica		49			11	49
Cuba			65			
El Salvador			149			
Peru	115	120	49	7	5	19
Suriname			18			
Trinidad		67				
Venezuela	232	294	165	62	60	40
North America	29,201	28,508	29,301	8,808	10,337	9,558
Canada	4,788	4,992	4,962	3,588	4,743	4,676
USA	24,413	23,516	24,339	5,220	5,594	4,882
Oceania	5,949	2,377	1,832	1,825	1,987	
Australia	4,202					
New Zealand	1,747	2,377	1,832	1,825	1,987	
Total countries	29	31	31	20	22	22
Total isolates serotyped	137,329	113,782	125,745	25,761	23,522	16,506

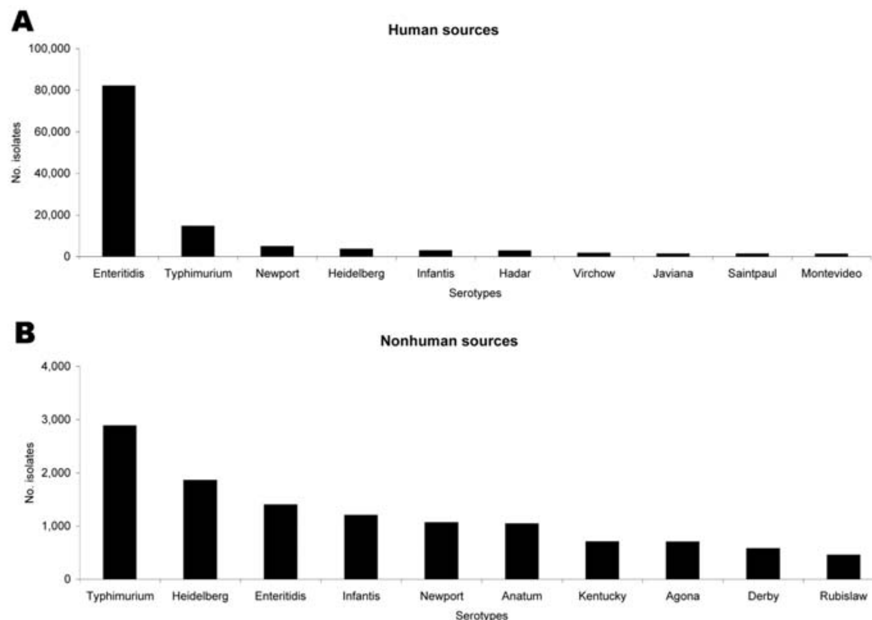

Figure 1. Number of *Salmonella* isolates reported by serotype worldwide in 2002.

In Asia, from 2000 through 2002, Japan, Korea, and Thailand together reported *S.* Enteritidis as the most common human serotype. *S.* Weltevreden was the second most common serotype in 2000 and 2001 but dropped to fourth in 2002, when it was surpassed by *S.* Rissen and *S.* Typhimurium. In 2002, *S.* Enteritidis accounted for 38% of human isolates but only 7% of nonhuman isolates. *S.* Anatum, *S.* Rissen, and *S.* Stanley were the most common nonhuman serotypes in Asia.

In Europe in 2002, *S.* Enteritidis accounted for most salmonellae among human isolates. This trend was constant from 2000 to 2002; *S.* Enteritidis accounted for 79% to 84% of isolates, followed by *S.* Typhimurium in second place and *S.* Hadar, *S.* Virchow, and *S.* Infantis alternating in the third to fifth places among the 8 countries that submitted data during the 3 years. Among nonhuman isolates, heterogeneity was greater; *S.* Infantis, *S.* Enteritidis, and *S.* Typhimurium together accounted for 72% of salmonellae in 2002.

In 2002 in Latin America and the Caribbean, *S.* Enteritidis was the most common serotype among human and nonhuman isolates. *S.* Typhimurium, *S.* Typhi, *S.* Montevideo, and *S.* Paratyphi B were also commonly observed among human isolates and *S.* Typhimurium, *S.* Senftenberg, *S.* Mbandaka, and *S.* Agona, among nonhuman isolates. During the 3-year period of interest, *S.* Enteritidis, *S.* Typhimurium, and *S.* Typhi were the 3 most commonly isolated serotypes among humans in the 5 countries that reported data every year.

In North America in 2002, *S.* Typhimurium was more common than *S.* Enteritidis among human isolates. *S.* Newport and *S.* Heidelberg also accounted for a sizeable proportion of the isolates. Among nonhuman isolates, a corresponding pattern emerges; *S.* Typhimurium, *S.* Heidelberg, and *S.* Newport were most common. *S.* Enteritidis was not reported among the 10 most common nonhuman serotypes. The relative ranking of serotypes did not change in the 3-year period; *S.* Typhimurium was the most common serotype in humans and nonhuman isolates from 2000 to 2002. In Oceania in 2000, the only year in which >1 country reported data, *S.* Typhimurium accounted for 62% of human *Salmonella* isolates, followed by *S.* Virchow and *S.* Enteritidis.

Some serotypes were reported among the 15 most common serotypes in only 1 region during the 3-year period and therefore were classified as region-specific serotypes. Africa was the only region to report *S.* Brancaster among nonhuman isolates. Asia was the only region to report *S.* Rissen (human), *S.* Panama and *S.* Stanley (nonhuman), and *S.* Weltevreden (human and nonhuman). Europe was the only region to report *S.* Blockley, *S.* Kisangani, *S.* Kottbus, *S.* Ohio, and *S.* Stanleyville from human isolates and *S.* Indiana and *S.* Isangi from nonhuman isolates. Latin America and the Caribbean was the only region to report *S.* Bardo, *S.* Muenster, and *S.* Rubislaw among human isolates. North America was the only region to report *S.* Javiana (human) and *S.* Muenster (nonhuman).

Discussion

S. Enteritidis is the most common *Salmonella* serotype in humans globally but especially in Europe, where it accounts for 85% of *Salmonella* cases, Asia (38%), and Latin America and the Caribbean (31%). The *S.* Enteritidis pandemic was first noted in the late 1980s and has been attributed to contaminated eggs (*11*). The proportion of *Salmonella* infections associated with this serotype seems

Table 2. Number and proportion of countries (N = 31) that ranked in the top 10 each of the 20 most common *Salmonella* serotypes among human isolates, 2002

Global rank	Serotype	Europe, n (%)	Asia, n (%)	Oceania, n (%)	Africa, n (%)	North America, n (%)	Latin America and Caribbean, n (%)	Total, n (%)
1	Enteritidis	8 (100)	4 (80)	1 (100)	4 (80)	2 (100)	7 (70)	26 (84)
2	Typhimurium	8 (100)	5 (100)	1 (100)	4 (80)	2 (100)	6 (60)	26 (84)
3	Newport	3 (38)	1 (20)	0	1 (20)	2 (100)	1 (10)	8 (26)
4	Heidelberg	2 (25)	2 (40)	0	0	2 (100)	2 (20)	8 (26)
5	Infantis	8 (100)	1 (20)	1 (100)	1 (20)	2 (100)	1 (10)	14 (45)
6	Hadar	6 (75)	3 (60)	0	3 (60)	2 (100)	0	14 (45)
7	Virchow	5 (63)	0	1 (100)	2 (40)	0	1 (10)	9 (29)
8	Javiana	0	0	0	0	1 (50)	2 (20)	3 (10)
9	Saintpaul	3 (38)	1 (20)	1 (100)	0	2 (100)	3 (30)	10 (32)
10	Montevideo	2 (25)	2 (40)	1 (100)	1 (20)	2 (100)	4 (40)	12 (39)
11	Agona	6 (75)	1 (20)	0	0	2 (100)	3 (30)	12 (39)
12	Oranienburg	0	0	0	0	2 (100)	1 (10)	3 (10)
13	Thompson	3 (38)	1 (20)	1 (100)	0	2 (100)	0	7 (23)
14	Typhi	1 (13)	2 (40)	1 (100)	4 (80)	1 (50)	5 (50)	14 (45)
15	Muenchen	0	0	0	0	1 (50)	0	1 (3)
16	Paratyphi B d-tartrate+	2 (25)	0	0	0	2 (100)	0	4 (13)
17	Braenderup	0	1 (20)	0	1 (20)	0	2 (20)	4 (13)
18	Blockley	2 (25)	0	0	0	0	0	2 (6)
19	Anatum	1 (13)	1 (20)	0	0	0	3 (30)	5 (16)
20	Weltevreden	0	2 (40)	0	0	0	1 (10)	3 (10)

to have increased over time. In 1995, 36% of salmonellae worldwide were *S*. Enteritidis, compared to 65% in 2002 (*8*).

S. Typhimurium has been 1 of the 2 most frequent serotypes in humans since 1990 (*8*). Since *S*. Enteritidis and *S*. Typhimurium are so common, additional subtyping methods, including phage typing, antimicrobial susceptibility testing, and pulsed-field gel electrophoresis (PFGE), are needed to identify clusters of infection from the same source. WHO Global Salm-Surv includes antimicrobial susceptibility testing training in all regional courses and has introduced phage typing in the Eastern European region course. The country databank could include data from such subtyping efforts. PFGE subtyping data are exchanged in North American between PulseNet USA and PulseNet Canada (*12*). PulseNet International is an affiliate member of WHO Global Salm-Surv, and the networks are coordinating their efforts to ensure synergy.

S. Typhi is a pathogen of concern in the developing world, especially Asia (*13*). However, in our analyses, *S*. Typhi was the ninth most frequent serotype in Asia in 2002. The Asian countries that contributed to the country

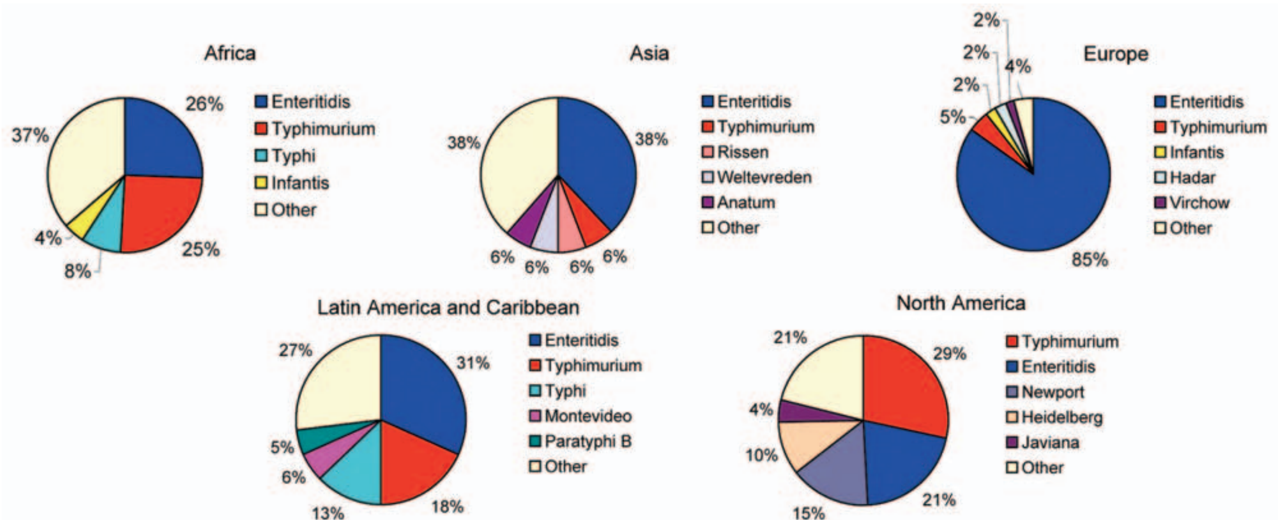

Figure 2. Proportion of most common serotypes of reported human *Salmonella* isolates by region, 2002.

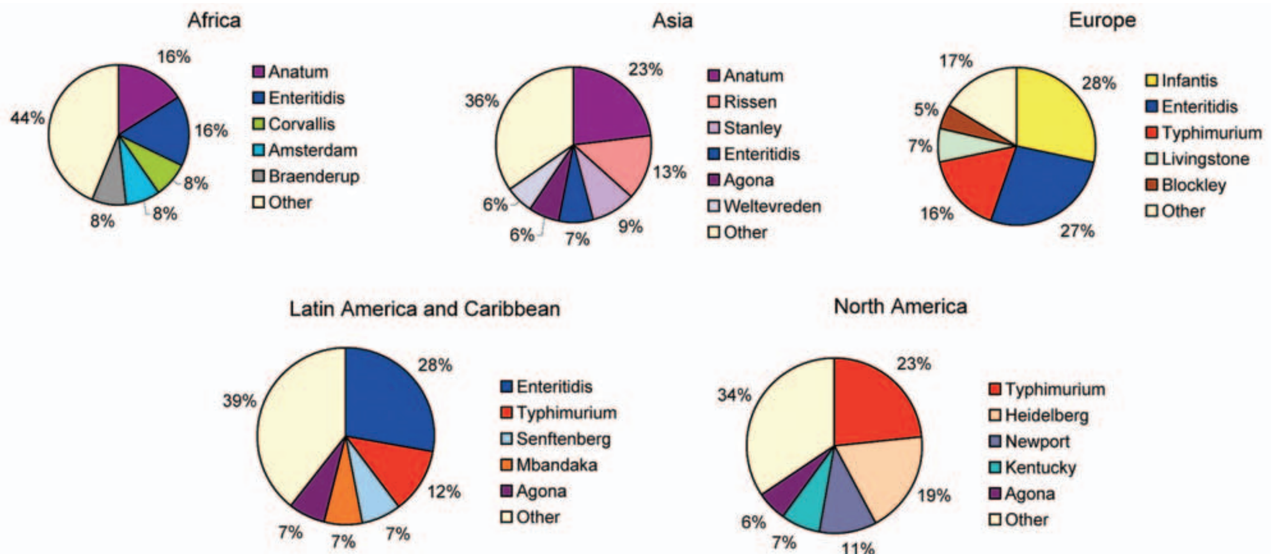

Figure 3. Proportion of most common serotypes of reported nonhuman *Salmonella* isolates by region, 2002.

databank did not include many of the developing countries in south-central and Southeast Asia, where *S.* Typhi is still highly prevalent. *S.* Typhi was the sixth most frequent serotype globally in 1995 and was decreasing in relative importance (*8*). That trend seems to have continued; *S.* Typhi ranked 14th globally in 2002. *S.* Typhi has no animal reservoir, which makes it susceptible to improvements in hygiene and sanitation seen in many regions of the world, such as Latin America and the Caribbean.

The distribution of nonhuman serotypes is more heterogeneous than that of human serotypes. The same serotypes appear among the top 5 in human and nonhuman sources, although in a different order. *S.* Enteritidis is only the third most common serotype among nonhuman sources. In 2002, it was not reported at all among the 10 most common nonhuman serotypes from North America. This finding partly reflects the capacity of *S.* Enteritidis to contaminate eggs in low numbers and the difficulty of isolating it from food or the environment. Moreover, in North America, few samples from eggs are submitted for routine testing. For example, in the United States, routine testing of eggs is not required, whereas routine testing for salmonellae is required of meat and poultry plants. As eggs are frequently used in foods that do not undergo heat treatment (e.g., pastries, homemade ice cream, and mayonnaise) and are widely distributed, this food contamination has a substantial effect on public health.

The country databank contains far fewer nonhuman than human serotypes, possibly because more participating laboratories are human national reference laboratories, fewer countries have formal nonhuman surveillance, and some countries may be less likely to share nonhuman data because of trade concerns. In 2001 and 2002, 15 of 22 countries reported nonhuman isolates by source. Food serotypes were reported from most countries (11 in 2001 and 12 in 2002), followed by animal serotypes (7 countries in 2001 and 10 in 2002). Most isolates serotyped were from animals (66%), followed by food (29%), feed (3%), and the environment (2%). The reporting of *S.* Weltevreden from the environment, feed, animals, food, and humans in Southeast Asia is an example of how the country databank can be used to track *Salmonella* serotypes along the food chain.

Many serotypes are restricted to a single region of the world. This finding may reflect an ecologic niche or a local food source that is not exported. A number of such examples have been reported in the past, such as *S.* Marina associated with marine iguanas from South America found in the United States and *S.* Tilene in imported African pygmy hedgehogs in the United States and Canada (*14–16*). The country databank is uniquely placed to allow countries to observe this phenomenon. Investigators have reported infections of *S.* Javiana associated with exposure to wild amphibians in a confined area in the southeastern United States (*17*). According to the country databank, *S.* Javiana is only reported among the 15 most common serotypes in the United States. In 2001, the WHO Global Salm-Surv country databank helped confirm that *S.* Weltevreden was largely restricted to Southeast Asia. A survey of Southeast Asian laboratories showed that items most frequently associated with this serotype include seafood, water, and Asian vegetables (*18*). In the same region, *S.* Rissen has increased in both human and nonhuman sources (*19*). The country databank allows countries to become aware that a common serotype in their country may be rare elsewhere in the world, leading to hypothesis generation in outbreaks

and studies to understand the sources of disease. Countries that report a large number of isolates to the country databank, such as North American and European countries, typically do not report rare serotypes because these would not rank in their top 15, thus limiting the ability to track rare serotypes in these countries.

Countries with fewer resources may lack complete antisera kits necessary to identify certain serotypes, which would lead to underreporting. For example, although we assume that *S.* Enteritidis human infections occur globally, a number of countries in Asia, Africa, and Latin America and the Caribbean did not report this serotype in their top 10 (Table 2). Lack of resources can also cause misclassification of serotypes. For example, *S.* Paratyphi B was reported to be among the most common serotypes in Latin America and the Caribbean. However, some countries in the region lack the capacity to differentiate between *S.* Paratyphi B and *S.* Paratyphi B tartrate var. Java.

In general, industrialized countries are more likely to regularly contribute to the country databank and to report more isolates. The results are therefore biased towards the industrialized world. However, the country databank lacks data from many Western European countries. Twenty-four European countries report human *Salmonella* serotype results annually to Enter-net, a European-based surveillance network for gastrointestinal infections, as compared to only 14 reporting to WHO Global Salm-Surv from 2000 to 2002. A review of recent Enter-net data confirms that *S.* Enteritidis is by far the most commonly isolated serotype in Europe but at a lower proportion than that reported to WHO Global Salm-Surv; 68%–71% of Enter-net *Salmonella* isolates were *S.* Enteritidis from 2000 to 2002 (Ian Fisher, pers. comm.) (*20*). A recent agreement between WHO Global Salm-Surv and Enter-net will lead to routine electronic sharing of data between the 2 systems to improve efficiency and representativeness (Henrik Wegener, pers. comm.).

Serotypes reported by a region are not necessarily circulating locally and may have been imported through travel or traded foods. Intraregional comparisons are limited by the fact that case definitions and surveillance systems vary between countries. The country databank does not collect the source of isolation. Some countries may report salmonellae isolated from both blood and stool and others from stool only. The low number of isolates and countries reporting nonhuman data and the pooling of food, animal, environmental, and feed sources hamper further analysis of nonhuman data. Some regional results may not be representative, since some regions have few countries reporting data to the country databank (e.g., Africa).

Some countries may not submit data to the country databank because of concern regarding international trading of food. Others do not have the supplies or training necessary to conduct serotyping. WHO Global Salm-Surv training courses were launched in Southeast Asia in 1999 and expanded to South America and the Middle East in 2000; China, Central America, and the Caribbean in 2001; and West Africa and Eastern Europe in 2002 (www.who.int/salmsurv). All participating countries were initially provided with antisera to conduct serotyping. WHO Global Salm-Surv established an external quality assurance system (EQAS) in 2000 to assess the accuracy of serotyping and antimicrobial susceptibility testing among member national reference laboratories. From 2000 to 2002, the number of laboratories participating in EQAS increased from 44 to 117, and the capacity to correctly serotype 8 *Salmonella* isolates improved from 76% to 90% of participating laboratories (*21*). The increased reporting of certain serotypes during the study period may be due to capacity improvement and increased participation in the country databank as well as real changes in the epidemiologic features of salmonellae. For example, *S.* Montevideo was first reported in Africa in 2001. In 2002, Tunisia participated in the country databank for the first time and reported *S.* Montevideo in its top 15 (increased participation). Oceania started reporting *S.* Montevideo in its top 15 in 2002 for the first time since participating in the country databank (change in epidemiologic features).

Data are not entered into the country databank in a timely enough way to detect international outbreaks. However, regularly monitoring the data can allow emerging trends in regional and international *Salmonella* epidemiology and region-specific serotypes to be detected. This information in turn leads to hypothesis generation, studies, and international collaboration to improve control of salmonellae in the long term. Examples of such work include the surveillance of *Salmonella* serotypes and antimicrobial drug resistance in South America, China, and the Democratic Republic of Congo; the assessment of risk factors for and drug resistance of *S.* Weltevreden in Southeast Asia; and the molecular characterization of *S.* Corvallis isolates from Bulgaria, Thailand, and Denmark (*18,22–26,* F. Aarestrup, pers. comm.).

The WHO Global Salm-Surv Country Databank is a valuable resource for international *Salmonella* surveillance. Past attempts to characterize *Salmonella* serotype distribution globally have either not been widely accessible or relied on irregular surveys of laboratories (*8,11*). Trends in global *Salmonella* epidemiology can now be updated and followed across regions and over time. In an era when most national institutions have access to the Internet, using a Web-based data collection tool is both feasible and practical. The data are immediately and publicly accessible for viewing and analysis (www.who.int/salmsurv). The results have several limitations in terms of representativeness and comparability but can be used to follow trends, generate

hypotheses, and assess the effect of major interventions. This surveillance is a step toward improving the understanding and control of salmonellae worldwide.

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Bartonella Spp. in Pets and Effect on Human Health

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Among the many mammals infected with *Bartonella* spp., pets represent a large reservoir for human infection because most *Bartonella* spp. infecting them are zoonotic. Cats are the main reservoir for *Bartonella henselae*, *B. clarridgeiae*, and *B. koehlerae*. Dogs can be infected with *B. vinsonii* subsp. *berkhoffii*, *B. henselae*, *B. clarridgeiae*, *B. washoensis*, *B. elizabethae*, and *B. quintana*. The role of dogs as an important reservoir of *Bartonella* spp. is less clear than for cats, because domestic dogs are more likely to be accidental hosts, at least in nontropical regions. Nevertheless, dogs are excellent sentinels for human infections because a similar disease spectrum develops in dogs. Transmission of *B. henselae* by cat fleas is better understood, although new potential vectors (ticks and biting flies) have been identified. We review current knowledge on the etiologic agents, clinical features, and epidemiologic characteristics of these emerging zoonoses.

Bartonella spp. are fastidious, hemotropic, gram-negative bacteria that are mainly transmitted by vectors. Among the 11 species or subspecies known or suspected to be pathogenic for humans, 6 have been isolated from pet dogs and cats (Table 1). Domestic cats are the principal reservoir for *Bartonella henselae*, the main agent of cat-scratch disease (CSD); *B. clarridgeiae*, which has been suspected in a few cases of CSD; and *B. koehlerae*, recently reported as the cause of human endocarditis (*1,4*). Domestic dogs could be one of the reservoirs for *B. vinsonii* subsp. *berkhoffii* (reported as *B. v. berkhoffii* thereafter) because as it can cause prolonged bacteremia in this species (*5,6*). Dogs can also be infected with *B. henselae*, *B. clarridgeiae*, *B. washoensis*, and *B. elizabethae* (*2*). Recently, 2 cases of endocarditis caused by *B. quintana* were diagnosed (P. Kelly et al., unpub. data). As with human disease, the clinical spectrum of *Bartonella* infection in dogs is expanding (*2*). Fleas play a major role in the transmission of feline *Bartonella* (*7*), but other potential vectors, such as ticks and biting flies have been recently identified to harbor *Bartonella* DNA, including *B. henselae* (*8,9*). This article provides an update on the etiologic agents, new clinical features, and evolving epidemiologic characteristics of these emerging zoonoses. We will not discuss the diagnosis, treatment, and prevention of *Bartonella* infections, as several recent review articles have been written on this subject (*1,2,10*).

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Feline *Bartonella* Species

B. henselae

Since the first isolation of *B. henselae* from a domestic cat in the early 1990s, several studies have been conducted worldwide to determine the importance of cats as a reservoir of this bacterium (reviewed in [*2*]). Prevalence of infection varies considerably among cat populations (strays or pets) with an increasing gradient from low in cold climates (0% in Norway) to high in warm and humid climates (68% in the Philippines) (*2*). At least 2 genotypes have been identified and designated Houston-1 (type I) and Marseille (previously BATF) (type II) (*1,2*). The respective prevalence of these 2 genotypes varies considerably among cat populations from different areas. *B. henselae* type Marseille is the dominant type in cat populations in the western United States, western Europe (France, Germany, Italy, the Netherlands, United Kingdom), and Australia, whereas type Houston-1 is dominant in Asia (Japan and the Philippines) (reviewed in [*2*]). However, within a given country, the prevalence may also vary among cat populations. For instance, in France, Marseille type was the most common type in cats from the Nancy and Paris areas, whereas type Houston-1 was the main genotype in cats from Lyon or Marseille (references cited in [*2*]). However, a few studies in western Europe and Australia have reported that most human cases of CSD were caused by *B. henselae* type Houston-1, despite the fact that type Marseille was found to be the dominant type in the cat population, which suggests that type Houston-1

Table 1. Species and subspecies of *Bartonella* that are confirmed or potential human pathogens

Bartonella sp.	Primary reservoir	Vector	Accidental host	Reference
B. bacilliformis	Human	Sandfly (*Lutzomia verrucarum*)	None	(*1,2*)
B. quintana	Human	Body louse (*Pediculus humanis*)	Cat, dog, monkey	(*1–3*, P. Kelly et al., unpub. data)*
B. elizabethae	Rat (*Rattus norvegicus*)	Oriental rat flea (*Xenopsylla cheopis*)	Human, dog	(*2*)
B. grahamii	Wild mice (*Clethrionomys glareolus, Microtus agrestis, Apodemus flavicollis*)	Rodent fleas	Human	(*1,2*)
B. henselae	Cat (*Felis catus*)	Cat flea (*Ctenocephalides felis*)	Human, dog	(*1,2*)
B. clarridgeiae	Cat	Cat flea	Human?, dog	(*1,2*)
B. koehlerae	Cat	Cat flea	Human	(*2,4*)
B. vinsonii subsp. *berkhoffii*	Coyote (*Canis latrans*), dog (*C. familiaris*)	Unknown (ticks?)	Human	(*5,6*)
B. vinsonii subsp. *arupensis*	White-footed mouse (*Peromyscus leucopus*)	Unknown (fleas?, ticks?)	Human	(*1,2*)
B. washoensis	California ground squirrel (*Spermophilus beecheyii*)	Unknown (fleas?)	Human, dog	(*2*)
B. alsatica	Rabbit	Unknown (flea?)	Human	(D. Raoult, pers. comm.)

*Also reported by O'Rourke LG, Pitulle C, Hegarty BC, Kraycirik S, Killary KA, Grosenstein P, et al. *Bartonella quintana* in cynomolgus monkey (*Macaca fascicularis*). Emerg Infect Dis. 2005;11:1931–4.

strains could be more virulent to humans (*2*). Cats are usually bacteremic for weeks to months, but some cats have been reported to be bacteremic for >1 year. Young cats (≤1 year) are more likely than older cats to be bacteremic (*11*), and stray cats are more likely to be bacteremic than pet cats (*1,2*).

The clinical description of CSD was first reported in France by Debré et al. in 1950, but the etiologic agent was identified only in 1992 (*1,2,6*). The annual number of cases in the United States has been estimated to be between 22,000 and 24,000, with ≈2,000 cases that require hospitalization, and thousands of cases may occur yearly in Europe. In various studies, the seroprevalence of antibodies to *B. henselae* in healthy persons has ranged from 3.6% to 6% (Table 2) and could be higher in some specific population groups, such as veterinarians, children, or elite orienteers (orienteering is a sport in which participants compete to find points in the landscape using a map and compass). Table 2 gives comparative *B. henselae* seroprevalence data for cat and healthy human populations from selected countries, which suggests that seroprevalence is low in both cats and humans at northern latitudes and increases in warmer climates (*11–24*). Such data are informative and cannot exclude possible serologic cross-reactivity with some other *Bartonella* spp.

Despite the fact that *B. henselae* infection can cause meningitis and encephalitis, only 1 case of a fatal infection has been reported (*5*). CSD is more frequently observed in persons <20 years of age and in persons who own a young cat (<1 year of age, especially if this cat is infested with fleas) or in persons who have been scratched or bitten by a cat (*1,2,6*). In immunocompetent persons, CSD is mainly characterized by a benign regional lymphadenopathy. Usually after a cat scratch, a papule and then a pustule develop within 7 to 12 days at the injection site, followed by a regional lymphadenopathy (usually involving a single lymph node) 1–3 weeks later that can persist for few weeks to several months. Low-grade fever, malaise, and aching are often reported; in some instances, headache, anorexia, and splenomegaly can occur. Abscessed lymph nodes are reported occasionally. In 5% to 9% of CSD patients, atypical manifestations may develop, including Parinaud oculoglandular syndrome, encephalitis, endocarditis, hemolytic anemia, hepatosplenomegaly, glomerulonephritis, pneumonia, relapsing bacteremia, and osteomyelitis.

On the basis of serologic testing or polymerase chain reaction (PCR), several recent publications have associated *B. henselae* with uveitis, focal retinal phlebitis, neuroretinitis, retinal and optical nerve neovascularization, and retinal artery and vein occlusions. Neurologic forms are rare, and patients usually completely recover within 1 year without sequelae. Hepatosplenomegaly and osteolytic bone lesions have been described in persons seropositive for *B. henselae*. Pseudotumoral lesions involving the mammary glands, the liver, or the spleen and, recently, glomerulonephritis and cases of monoclonal and biclonal gammopathy have also been associated with *B. henselae* antibodies. Cases of prolonged fever without adenopathy, chronic fatigue, hemolytic anemia, thrombocytopenic purpura, Henoch-Schönlein purpura syndrome, pleuritis, pneumonia, and even paronychia have been reported in patients who were seropositive for *B. henselae* (*1,2*). Usually, these clinical manifestations disappear in a few weeks to a few months. Bacteremia is rarely detected in immunocompetent persons. Several cases of endocarditis

Table 2. *Bartonella henselae* seroprevalence in various cat and human populations from selected countries*

Country	Cat seroprevalence (%)			Human seroprevalence (%)		
	Stray	Pet	Reference	Healthy	Other	Reference
Sweden	NA	1	(*19*)	1	NA	(*12*)
Japan	NA	8.8–15.1; northern, 0–2; central 10.9–12.6; southern, 18–24	(*20*)	4.5	11.0–15.0 (veterinarians)	(*13,14*)
United States	81	27.9	(*11,21*)	3.6–6	7.0 (veterinarians)	(*15*)
Thailand	27.6†	NA	(*22*)	5.5	NA	(*16*)
Italy	39.0	43.5	(*23*)	NA	8.5–61.6 (children)	(*17*)
Jordan	NA	32.0	(*24*)	NA	NA	(*18*)

*NA, not available.
†Prevalence of bacteremic cats; no data available on seroprevalence.

have been associated with *B. henselae* infection, most frequently in persons with preexisting valvular lesions. Besides *B. henselae*, most human cases of *Bartonella* endocarditis are caused by *B. quintana*, but a few cases of endocarditis or myocarditis have been associated with *B. elizabethae* (1 case), *B. vinsonii berkhoffii* (1 case), *B. vinsonii arupensis* (1 case), *B. koehlerae* (1 case), *B. washoensis* (1 case), and *B. alsatica* (1 case) (Table 3).

In immunocompromised patients, *B. henselae* infection can cause prolonged fever, prolonged bacteremia, or both (*1,2,6*). Bacillary angiomatosis or peliosis is usually observed in highly immunocompromised persons (low CD4 count), who often are infected with HIV. Several severe infections have also been reported in organ transplant recipients (*1,2*).

The clinical spectrum of the infection in cats has not been fully investigated, but naturally infected cats primarily seem to be healthy carriers of the bacterium (*1,2,6*). However, cases of uveitis and rare cases of endocarditis have been molecularly associated with infection caused by *B. henselae*. Seropositive cats were more likely to have kidney disease and urinary tract infections, stomatitis, and lymphadenopathy. In experimentally infected cats, fever, lymphadenopathy, mild neurologic signs, and reproductive disorders have been reported.

B. clarridgeiae

B. clarridgeiae was first isolated in the United States from the pet cat of an HIV-positive patient (*25*). This *Bartonella* sp. has been less frequently isolated from domestic cats than *B. henselae* because it appears to be more difficult to isolate and is unevenly distributed in cat populations worldwide. A *B. clarridgeiae* prevalence of 17% to 36% among all *Bartonella* isolates was reported in studies conducted in France, the Netherlands, the Philippines, and Thailand (*2,22*). However, *B. clarridgeiae* represented ≤10% of all isolates from domestic cats in the southeastern United States, Japan, or Taiwan (*2*) and has never been isolated in studies conducted in Europe, Australia, and North America (*2*). No specific pathologic features have been associated with natural infection in cats. However, in experimentally coinfected cats (*B. henselae* type II and *B. clarridgeiae*), clinical signs were minimal, and gross necropsy results were unremarkable, but histopathologic examination showed peripheral lymph node hyperplasia, splenic follicular hyperplasia, lymphocytic cholangitis/pericholangitis, lymphocytic hepatitis, lymphoplasmacytic myocarditis, and interstitial lymphocytic nephritis (*26*). In humans, *B. clarridgeiae* has never been isolated or detected by molecular methods. However, *B. clarridgeiae* could be a minor causative agent of CSD, as the presence of *B. clarridgeiae* antibodies were reported in a suspect case of CSD and in a patient with a chest-wall abscess (reviewed in [2]). Furthermore, anti-flagella (FlaA)–specific antibodies against *B. clarridgeiae* were detected by immunoblotting in 28 (3.9%) of 724 patients with lymphadenopathy but in none of 100 healthy controls. However, substantial cross-reactivity between *B. henselae* and *B. clarridgeiae* detected by indirect fluorescence antibody assay was noted in human sera in a recent study from Japan (*2*).

B. koehlerae

B. koehlerae is a *Bartonella* sp. that has rarely been isolated from domestic cats worldwide, as it is a very fastidious bacterium (*2,4*). Until recently, it had been isolated only from 2 cats in California and 1 cat in France (*2,4,27*). The first human case of *B. koehlerae* endocarditis was reported from Israel in 2004 (*2*). Furthermore, these authors were able to isolate *B. koehlerae* from a bacteremic stray cat from that country.

B. quintana and *B. bovis*

A few suspect cases of CSD and cases of bacillary angiomatosis or endocarditis have been associated with *B. quintana,* for which the only risk factor identified was a contact with cats or cat fleas (*3*). Furthermore, the identification of *B. quintana* DNA in cat fleas (*28*) and recently in the dental pulp of a cat (*3*) has raised the question as to whether cats might be a possible source of human infection. However, *B. quintana* has not yet been isolated from naturally infected cats anywhere in the world where epidemiologic studies have been conducted to detect *Bartonella*-bacteremic cats. Similarly, 2 cats infected with

Table 3. Clinical aspects of *Bartonella* infections in humans and dogs

	Symptoms	
Bartonella sp.	Humans	Dogs
B. clarridgeiae	Cat-scratch disease	Endocarditis, lymphocytic hepatitis
B. elizabethae	Endocarditis, neuroretinitis	Lethargy, anemia, weight loss
B. henselae	Cat-scratch disease, endocarditis, bacillary angiomatosis, peliosis hepatis, granulomatous hepatitis, pseudotumoral lesions, arthritis, arthralgia, osteomyelitis, nodules, erythema, cutaneous petechiae, uveitis, neuroretinitis, purpura (Henoch-Schönlein), glomerulonephritis, perionyxis, periodontitis	Granulomatous hepatitis, peliosis hepatis, epistaxis
B. grahamii	Neuroretinitis, bilateral retinal artery branch occlusions	Not diagnosed in dogs
B. koehlerae	Endocarditis	Not diagnosed in dogs
B. vinsonii subsp. *arupensis*	Bacteremia, fever, arthralgia, neurologic disorders, endocarditis	Not diagnosed in dogs
B. vinsonii subsp. *berkhoffii*	Endocarditis	Endocarditis, myocarditis, arrhythmia, uveitis, choroiditis, limping, splenomegaly, polyarthritis, epistaxis
B. washoensis	Fever, myocarditis	Endocarditis
B. quintana	Fever, bacteremia, endocarditis, bacillary angiomatosis	Endocarditis

B. quintana did not become bacteremic but seroconverted (*29*). Subsequently, both cats became bacteremic when challenged with *B. henselae*.

A few cases of *B. bovis* (formerly *B. weissii*) infections have been reported in cats from Illinois and Utah in the United States (*1*). The epidemiologic role of cats for this organism is still unknown.

Dogs as Sentinels for Human Infections?

Dogs can be infected with *B. v. berkhoffii*, *B. henselae*, *B. clarridgeiae*, *B. washoensis*, *B. elizabethae*, and *B. quintana* (*2*, P. Kelly et al., unpub. data). However, the role of dogs as a major reservoir of *Bartonella* spp. is not clear. Current evidence suggests that domestic dogs are more likely to be accidental hosts of various *Bartonella* spp., at least in nontropical regions. Nevertheless, domestic dogs could be one of the reservoirs for *B. v. berkhoffii*, as it causes prolonged bacteremia in this species (*5,6*). The epidemiologic situation is quite distinct between tropical areas where several studies have shown a high prevalence of *B. v. berkhoffii* antibodies, especially in stray dogs, and more northern latitudes, where very low antibody prevalence has been detected in domestic dogs, especially among pets. In sub-Saharan Africa, seroprevalence of 26% in dogs in Senegal and up to 65% in native dogs from Sudan has been reported (*1*). In North Africa, we found that 38% of 147 dogs from Morocco were seropositive for *B. v. berkhoffii* (*30*). In 113 dogs from the Reunion Island, in the Indian Ocean, a seroprevalence of 18% was reported in stray dogs, whereas only 3% of dogs examined at veterinary clinics were seropositive, and no dog was bacteremic (*31*). In Thailand, 38% of sick dogs who exhibited fever, anemia, or thrombocytopenia were seropositive for *B. v. berkhoffii* (*1*). On the contrary, studies in the United States and Europe reported a seroprevalence of <5% in domestic dogs; selected dog populations were at higher risk, including rural dogs and government working dogs (*2*). However, concerns about false-positive results in animals should be raised, as specificity and sensitivity of the tests for dogs have not been fully evaluated. In California, *B. v. berkhoffii* has rarely been isolated from domestic dogs or detected by PCR, whereas coyotes (*Canis latrans*) appear to be a reservoir of this pathogen, as 35% of the coyotes tested in California were seropositive, and 28% of the coyotes tested within a highly disease-endemic region of California were bacteremic (*2*).

In domestic dogs, *B. v. berkhoffii* is a cause of endocarditis (*6*) and, as in humans, the clinical spectrum of the infection attributed to this organism is expanding. *B. v. berkhoffii* is now associated with cardiac arrhythmias, endocarditis and myocarditis, granulomatous lymphadenitis, granulomatous rhinitis, and epistaxis (*6,32*). In both humans and dogs, *Bartonella*-associated cases of endocarditis usually involve the aortic valve and are characterized by massive vegetative lesions (*33*). Based on serologic evidence, infection with *B. v. berkhoffii* may also cause immune-mediated hemolytic anemia, neutrophilic or granulomatous meningoencephalitis, neutrophilic polyarthritis, cutaneous vasculitis, and uveitis in dogs (*2*).

Some other *Bartonella* spp. have infrequently been isolated from domestic dogs. *B. clarridgeiae* and *B. washoensis* were isolated from cases of endocarditis (*1,2*), and *B. henselae* was isolated for the first time from a dog from Gabon (*34*). In the Gabon study, *B. clarridgeiae* was isolated from 5 of 258 dogs tested (1.9%), which suggests a possible reservoir role for this *Bartonella* sp. in Africa (*34*). *B. henselae*, *B. elizabethae*, and *B. clarridgeiae* DNA has also been detected from a few sick dogs with various clinical abnormalities (Table 3) (*1,2,6*). Endocarditis caused by *B. quintana* was recently diagnosed in a dog from the United States and a dog from New Zealand (P. Kelly et al., unpub. data). Two recent studies reported a

B. henselae antibody prevalence of 10% in healthy dogs in the eastern United States (*35*) and a prevalence of 14% of dogs in Zimbabwe (*36*). A much higher prevalence (27%) in sick dogs from the eastern United States was reported (*35*), which contrasts with the low *B. henselae* seroprevalence (<2%) in dogs examined at a university teaching hospital in northern California (*37*). A case-control study conducted on 305 dogs (102 dogs seropositive for *B. henselae*, *B. v. berkhoffii*, or *B. clarridgeiae* and 203 seronegative dogs) suggested an association between the seropositive status and lameness, arthritis-related lameness, splenomegaly, and nasal discharge/epistaxis (*37*).

Unlike the domestic cat, for which clinical manifestations of natural infection is rarely documented, a wide range of clinical and pathologic abnormalities develop in dogs that are very similar to those observed in humans (*32*). Therefore, this species is an excellent sentinel and an important comparative model for human infections. To date, all *Bartonella* spp. identified in sick dogs are also pathogenic or potentially pathogenic in humans.

Beyond the Fleas: New Emerging Vectors

The primary mode of transmission of *B. henselae* to humans is through a cutaneous trauma caused mainly by the scratch of a cat. Transmission is less likely to occur by cat bite; shedding of *B. henselae* in cat saliva has not been clearly documented. The possibility of direct transmission of *B. henselae* to humans by the cat flea is something that has not been proven experimentally and is mainly hypothetical. However, the presence of cat fleas (*Ctenocephalides felis*) is essential for the maintenance of the infection within the cat population (*6*). *B. henselae* has been shown to multiply in the digestive system of the cat flea and survive several days in the flea feces (reviewed in [*2*]). Experimentally, only cats inoculated with flea feces compared to those on which fleas were deposited in retention boxes or that were fed fleas became bacteremic (*38*). Therefore, the main source of infection appears to be flea feces that are infected by contaminated cat claws.

Beside the cat flea, new possible vectors have been suggested. *Bartonella* DNA, including *B. henselae*, has been detected in *Ixodes ricinus* ticks collected on humans (*9*) and in *I. scapularis* ticks collected in households of persons coinfected with *B. henselae* and *Borrelia burgdorferi* (reviewed in [*2*]). *B. quintana*, *B. henselae*, and *B. v. berkhoffii* DNA were also detected in questing *I. pacificus* ticks in California, and a few human cases of *B. henselae* infection were temporally related to a tick exposure in the United States (reviewed in [*2*]). Tick exposure was reported as a risk factor associated with CSD in humans (*39*). Similarly, tick exposure was determined to be a risk factor associated with *B. v. berkhoffii* seropositivity in dogs (*40*). Additional indirect support for ticks as vectors of *B. v. berkhoffii* in dogs relates to serologic or PCR evidence of concurrent infections with various tickborne organisms (*6,33*). The specific role of ticks in *Bartonella* transmission requires additional study, but several recent publications have reported a high prevalence of *Bartonella* spp. infection in ticks from various parts of the world. Finally, *B. henselae* type Marseille DNA was recently detected in a stable fly (*8*).

Conclusion

The number of zoonotic *Bartonella* species identified in the last 15 years has increased considerably. Pets have been identified as a notable reservoir of *Bartonella* species (i.e., cats and *B. henselae* or dogs and *B. v.* subsp. *berkhoffii* in the tropics) and may play an important role as source for human infection. Furthermore, domestic dogs may represent excellent sentinels for *Bartonella* infection because of the wide diversity of the *Bartonella* spp. identified in canines, all of which are human pathogens. A better understanding of the modes of transmission and vectors involved in dog bartonellosis is an urgent priority to implement appropriate parasite control measures for pets.

Dr Chomel is the director of the World Health Organization/Pan American Health Organization Collaborating Center on New and Emerging Zoonoses at the University of California, Davis. His research focuses on *Bartonella* infections in domestic animals and wildlife and their impact on human health.

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West Nile Virus Infections Projected from Blood Donor Screening Data, United States, 2003

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National blood donor screening for West Nile virus (WNV) RNA using minipool nucleic acid amplification testing (MP-NAT) was implemented in the United States in July 2003. We compiled national NAT yield data and performed WNV immunoglobulin M (IgM) testing in 1 WNV-epidemic region (North Dakota). State-specific MP-NAT yield, antibody seroprevalence, and the average time RNA is detectable by MP-NAT were used to estimate incident infections in 2003. WNV donor screening yielded 944 confirmed viremic donors. MP-NAT yield peaked in August with >0.5% of donations positive for WNV RNA in 4 states. Peak IgM seroprevalence for North Dakota was 5.2% in late September. The average time viremia is detectable by MP-NAT was 6.9 days (95% confidence interval [CI] 3.0–10.7). An estimated 735,000 (95% CI 322,000–1,147,000) infections occurred in 2003, with 256 (95% CI 112–401) infections per neuroinvasive case. In addition to preventing transfusion-transmitted WNV infection, donor screening can serve as a tool to monitor seasonal incidence in the general population.

After its identification in New York City in 1999, West Nile virus (WNV), a mosquitoborne flavivirus, emerged as a cause of neuroinvasive disease (meningitis, encephalitis, and acute flaccid paralysis) and febrile illness in the United States (*1–5*). Since 2000, a national surveillance system, ArboNET, has monitored WNV activity in mosquitoes, horses, and other animals, as well as cases of febrile illness and neuroinvasive disease in humans (*2*). Seroprevalence studies after epidemics indicate that febrile illness develops in ≈20% of infected persons, while neuroinvasive disease develops in <1% (*6,7*). On the basis of reported neuroinvasive cases and an estimated ratio of the number of infections to neuroinvasive cases, as of October 2004, a total of ≈1 million persons have been infected with WNV in the United States (*2*).

Evidence accumulated in 2002 that WNV could be transmitted by blood transfusion, culminating in 23 documented cases that year (*8–10*). In late 2002, the US Food and Drug Administration (FDA), US blood collection organizations, and test manufacturers began an accelerated program to implement nucleic acid amplification testing (NAT) of blood donors for West Nile viremia before the 2003 season (*11,12*). Assays were developed for use in a minipool-NAT format (i.e., samples of donations are pooled, and the pool is tested), similar to procedures now routinely used for blood screening for HIV-1 and hepatitis C virus (HCV) by NAT (*13*). In addition to minipool-NAT screening, several blood centers performed individual donation NAT screening in regions experiencing epidemic WNV activity to interdict donations with low-level viremia that could be missed by minipool-NAT (*14–18*).

Synthesis of blood donor screening data may provide an opportunity for public health surveillance in addition to ArboNET because of the large number of donations screened from a broad cross-section of the adult population. We report the combined results of WNV donor screening during the summer and fall of 2003 by America's Blood Centers (ABC) and the American Red Cross (ARC), which together collect and test ≈95% of US donations. In addition, WNV IgM and IgG testing was performed on donor specimens from 1 WNV-epidemic region

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to determine the proportion of donors with measurable antibody responses to WNV during 2003. We used this proportion, along with the minipool-NAT data from that region, to determine the average time during which WNV RNA was detectable by minipool-NAT. This time was combined with the minipool-NAT donor screening data for each state and US Census data to estimate the proportion of WNV-infected persons (seasonal incidence) in each state and the total number of incident infections nationwide in 2003.

Methods

Overview of Approach

Since July 2003, all blood donations have been screened for WNV RNA by NAT. If we assume that blood donation and WNV infection are independent events, the proportion of blood donors infected by WNV in 2003 (seasonal incidence of WNV in the blood donor pool) is a function of NAT yield and the average length of time that WNV RNA is detectable after infection. By measuring IgM antibodies in North Dakota blood donors shortly after the epidemic, we estimated the seasonal incidence for that region. After adding NAT screening yield data from the same donor population, we then estimated the length of time that WNV RNA is detectable by NAT.

We combined the length of time that RNA is detectable by NAT with NAT screening yield data by state to estimate state-specific and national WNV seasonal incidence in the blood donor and general population. Finally, by dividing the estimated number of infections in the general population in 2003 by the number of neuroinvasive disease cases reported to the national WNV surveillance system (ArboNET), we estimated the ratio of WNV infections to neuroinvasive disease cases.

Blood Donor Screening

US blood donations are screened for WNV RNA by using NAT assays on pools of 6 to 16 donations or on individual samples in high-incidence regions. In 2003, ≈96% of the screening was conducted on pooled samples. Additionally, 2 blood collection organizations (ARC; Blood Systems) retrospectively performed individual donation NAT on cryopreserved plasma from 36,269 donations in 5 states with substantial epidemics to ascertain the proportion of low-level viremia missed by minipool-NAT and to assess, through recipient lookback, the infectivity of units harboring low-level viremia (*15,16*). Viremic donations detected by individual donation NAT were included in this analysis to compile total NAT yield for 2003, but they were excluded from calculations used to project WNV infection in the general population for the following reasons: 1) inconsistent application of individual donation NAT screening around the country (*15,16*); 2) variable rate of detection of low-level viremia by individual donation NAT assays (*18*); and 3) fever and symptoms during the postseroconversion low-level viremia phase (unlike the asymptomatic minipool yield phase), which would result in self-deferral from donation and bias our projections.

West Nile Viremia in US Blood Donors: Geographic and Temporal Distribution

We combined 2 large databases consisting of all donations and WNV-confirmed donations obtained by state from July 1 (when most blood centers implemented WNV NAT screening) to October 31, 2003. The first database was derived from 72 of the 74 independent blood centers that constitute ABC, which collect nearly 50% of US donations (*14,19*). Data elements included total number of donations, donor state of residence, and the minipool and individual donation NAT confirmatory status of all donations collected from July to October 2003. Similar data were obtained from the ARC national donor database, which constitutes 45% of the US supply (*16*). Donations were classified as confirmed WNV NAT-positive if the index donation was reactive by NAT and 1) positive for IgM or by an alternative NAT procedure or 2) follow-up samples from donors were reactive on a NAT assay or were IgM-positive. The dataset included the subset of confirmed NAT-positive donations that were either originally detected by minipool-NAT, or had been detected by prospective individual donation NAT but were subsequently determined to be detectable by minipool-NAT (were reactive when retested at a 1:16 dilution using minipool-NAT). This extra testing ensured that seasonal incidence estimates were based on data obtained by using a comparably sensitive screening process across all regions of the United States and throughout the epidemic period. The proportion of confirmed positive donations identified by minipool-NAT was determined by month for each state, and ≈95% confidence intervals (CIs) around these minipool-NAT yield estimates were computed (*20*). The Epi Map component (Environmental Systems Research Institute, Redlands, CA, USA) of EpiInfo version 3.3 (Centers for Disease Control and Prevention [CDC], Atlanta, GA, USA) was used to display results graphically.

Estimate of Days West Nile Viremia Is Detectable by Minipool-NAT

To use NAT screening data to estimate state-specific WNV seasonal incidences, we first derived an estimate for the average length of time that RNA is detectable by minipool-NAT after infection occurs (TMP-NAT). TMP-NAT can be approximated if both the minipool-NAT screening yield and seasonal incidence of WNV are known (Appendices 1 and 2; available from http://www.bsrisf.org/

eid2006/app1.html and http://www.bsrisf.org/eid2006/app2.html). The seasonal incidence was estimated by measuring the peak WNV IgM prevalence observed in a particular region ≈3 weeks after the end of the region's first epidemic (http://www. bsrisf.org/eid2006/app1.html). Serologic data allowed us to evaluate both minipool-NAT yield and prevalence of IgM for each week from July to September 2003 and to identify peak IgM prevalence. The sum of the weekly minipool-NAT yield estimates divided by the peak IgM prevalence (our estimate of the 2003 seasonal incidence in North Dakota) was used to derive TMP-NAT (http://www.bsrisf.org/eid2006/app2.html). Approximate 95% CIs around peak IgM prevalence and TMP-NAT were calculated by assuming normal distributions with variances approximated by Taylor series (*21*).

WNV Seasonal Incidence

We assumed that WNV infection dynamics are similar in blood donors and in the general population. The monthly WNV incidence in each state for each month from July through October was derived by multiplying the monthly minipool-NAT yield by the number of days in each month and dividing by the average period of time during which RNA is detectable (TMP-NAT) (Appendix 3; available from http://www.bsrisf.org/eid2006/app3.html). Each state-specific seasonal WNV incidence was calculated by summing the 4 monthly WNV incidence estimates. To estimate 2003 WNV infections nationwide, we multiplied each state-specific seasonal WNV incidence by the corresponding July 1, 2003, population estimate from the US Census Bureau (*22*) and then summed over all states. An ≈95% CI around the estimated 2003 WNV infections nationwide was calculated by assuming a normal distribution with variance approximated by Taylor series (*21*).

Proportion of West Nile Infections Resulting in Neuroinvasive Disease

We then calculated the ratio of the estimated number of WNV infections nationwide and the total neuroinvasive disease cases reported to CDC (*23*). The standard error (SE) of this ratio is dependent on the SE of the total neuroinvasive disease cases (assumed to be Poisson distributed), the SE of TMP-NAT, and the SEs of state-specific minipool-NAT yield estimates (assumed to be binomially distributed) and was approximated by a Taylor series (*21*). We did not estimate the proportion of infections resulting in West Nile–related febrile illness because it is considerably underreported to ArboNET.

Approvals for Research on Human Subjects

The Investigational New Drug protocols, which included donor consent for WNV NAT screening and follow-up testing, were reviewed and approved by multiple institutional review boards and FDA. Institutional review board approval of this study protocol, including compilation of a national NAT yield database and anonymous IgM and IgG testing (http://www.bsrisf.org/eid2006/app1. html), was obtained from the University of California, San Francisco Committee for Human Research, and from Westat (Rockville, MD, USA).

Results

Overall, 944 confirmed West Nile viremic donors (0.02%) were identified by NAT screening among 4,585,573 donations from July 1 to October 31, 2003, at ARC and participating centers in ABC. These included 770 donations detected by minipool-NAT and 174 donations detected only as a result of prospective or retrospective individual donation NAT. The distribution of minipool-NAT and individual donation NAT yield by month is shown in Figure 1. Of the 191 viremic donations detected in July, only 2 were detected in the first week of July (both on July 6), and only 4 confirmed viremic donations were reported by ABC or ARC after October 31; thus, the July–October period composes virtually the entire 2003 epidemic. Geographically, the epidemic was most dramatic in the Central Plains states. The rate of WNV-infected donations exceeded 3 per 1,000 in Colorado in July and August and in 4 additional contiguous states in August (Figure 2).

North Dakota Data

As shown in Figure 3, minipool-NAT–confirmed positive donations were detected from July 13 to September 6, 2003, in the Bismarck and Minot regions of North Dakota, with minipool-NAT yield peaking at 1.4% (95% CI 0.4–2.3) in late August. IgM-confirmed positive donations were not observed in these same regions during the first 3

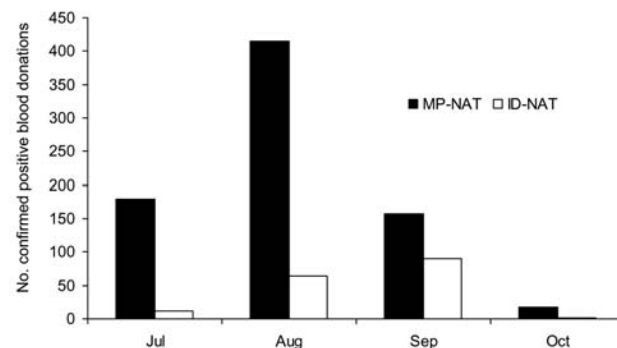

Figure 1. Yield of West Nile virus nucleic acid amplification test (NAT) screening of 4,585,573 donations at American Red Cross and America's Blood Centers (constituting ≈95% of US collections) from July 1 to October 31, 2003. A total of 944 confirmed viremic donations were identified, including 770 that were detectable by minipool-NAT and 174 detectable only by individual donation NAT. MP, minipool; ID, individual donation.

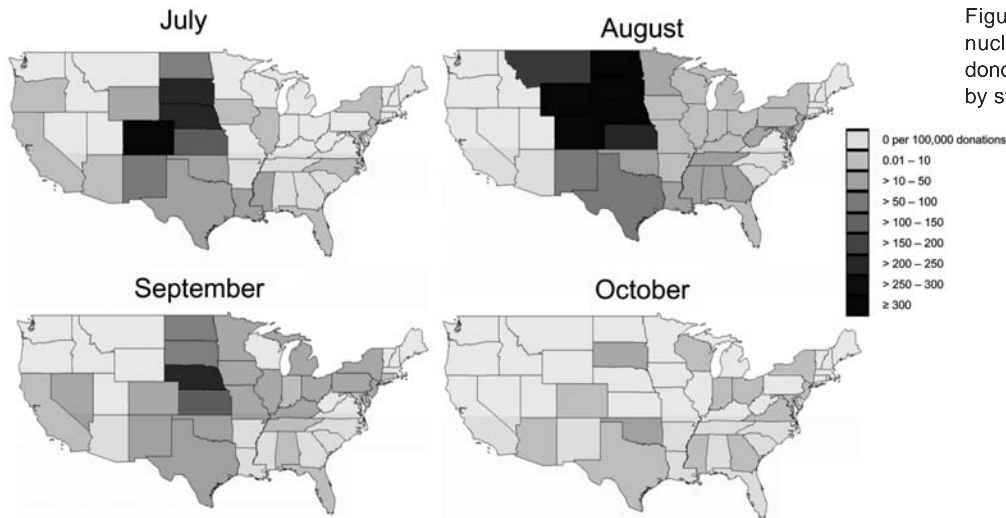

Figure 2. Yield of minipool–nucleic acid testing of blood donors for West Nile virus RNA by state and month, 2003.

weeks of July but were detected toward the end of July. IgM prevalence gradually increased thereafter and reached a plateau around September 7; ≈5% of donations were positive for IgM during most of September. The peak IgM prevalence was observed the last week of September (5.2%, 95% CI 3.0–7.4) and was similar to the IgG prevalence observed 9 months later in June 2004 (5.3%, 95% CI 3.9%–6.7%), when IgM prevalence had declined to 1.2%. Thus, the peak IgM weekly prevalence was assumed to be a good estimate of the seasonal incidence in this region. The average length of time viremia is detectable by minipool-NAT, TMP-NAT, was estimated to be 6.9 days (95% CI 3.0–10.7).

WNV Seasonal Incidence

The proportion of the population estimated to have become infected during 2003 in each state was 0%–4.9% (Figure 4A and Table). The highest proportions were observed in Nebraska (4.9%), Colorado (4.3%), North Dakota (4.1%), South Dakota (4.0%), Wyoming (3.5%), and Kansas (2.1%). Nationally, 735,000 persons (95% CI 322,000–1,147,000) were estimated to have been infected in 2003 (Table). Figure 4B shows the distribution of these infections by state. The greatest number of infections were located in Colorado, Texas, Nebraska, Kansas, the Dakotas, and to a lesser extent the states in the Midwest and Northeast, which had only moderate seasonal incidence but have large populations.

Reported WNV Neuroinvasive Disease Relative to Projected Infection Incidence

We compared the estimated proportion of the population infected with WNV to the proportion of WNV neuroinvasive disease cases reported to CDC for each state. Figure 5 shows that these proportions are highly correlated with one another. A total of 2,866 neuroinvasive WNV cases were reported nationally to CDC's ArboNET system in 2003 (Table). This total was compared to 735,000 persons nationally estimated to have been infected with WNV in 2003. Thus, an estimated 256 WNV incident infections occurred per reported neuroinvasive disease case (95% CI 112–401). An estimate of 353 infections per each reported neuroinvasive disease case (95% CI 190–516) was obtained by analyzing the North Dakota data separately, which had 94 reported neuroinvasive cases among an estimated 33,000 infections (5.2% peak IgM prevalence × the state population of 633,837).

Sensitivity Analyses

Two potential biases may have affected our estimated ratio of 256 WNV incident infections per reported neuroinvasive disease case. First, neuroinvasive cases may be underreported to ArboNET. A 20% underreporting of neuroinvasive cases to ArboNET alters the ratio to 205 WNV

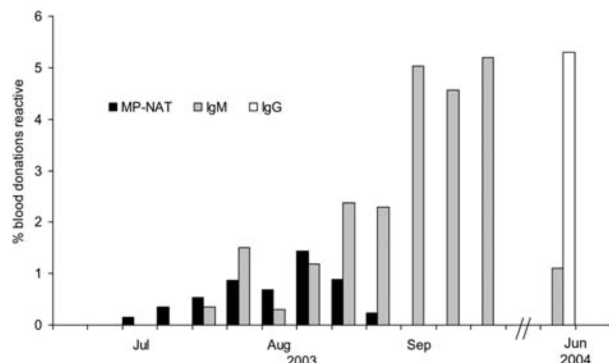

Figure 3. West Nile virus minipool—nucleic acid amplification testing (MP-NAT) yield and immunoglobulin M (IgM) and IgG seroprevalence estimates for North Dakota, during and ≈8 months after the 2003 epidemic period.

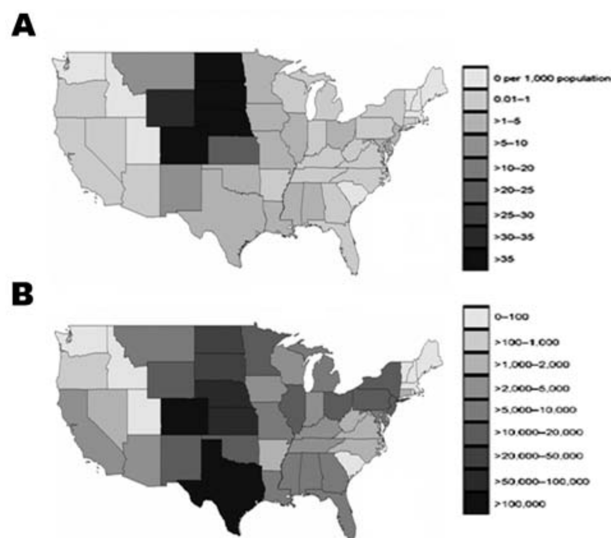

Figure 4. A) Projected number of West Nile virus (WNV) infections per 1,000 persons. B) Estimated total number of WNV infections per state during 2003 epidemic season.

incident infections per reported neuroinvasive disease case (95% CI 90–320). Second, blood donors may underrepresent infections in the general population because prospective donors who are WNV-infected may self-defer or be deferred from donating. If the number of infections in the general population was underestimated by 20%, the ratio of WNV incident infections per reported neuroinvasive disease case would be 320 (95% CI 140–501).

Discussion

This project, which collected and analyzed WNV screening data for 95% of US blood donations during the 2003 epidemic, identified 944 viremic donations among 4.6 million donations screened from July 1 to October 31. The number of viremic donors identified in 2003 is a slight underestimate since viremic donors identified by participating ABC and ARC centers outside the study time frame and viremic donors identified at nonparticipating collection centers and the military blood program were not included. The ABC and ARC data indicate that ≈1,000 West Nile viremic donors were identified in the United States in 2003 by prospective NAT screening, and consequently ≈1,500 potentially infectious blood components were interdicted before transfusion (*24*). This yield is particularly remarkable when compared with NAT screening for HIV-1 and HCV, which identified only 12 HIV and 170 HCV-infected antibody-negative donations among ≈39 million donations screened in the first 4 years of testing (*13*).

One goal of this project was to monitor the geographic and temporal distribution of WNV in the US blood donor population. We documented rates of viremic blood donors exceeding 3 per 1,000 donations in some states during the peak of the 2003 epidemic. The proportion of confirmed positive donations identified by minipool-NAT paralleled the neuroinvasive case reports in each state. Blood donor NAT screening data are useful for population surveillance because the testing has a rapid turnaround time, infections are identified soon after WNV acquisition, many of these infections remain asymptomatic, and typically those in whom symptoms develop are identified before illness onset. Communication of WNV donor screening data from blood centers to state and county health departments thus augments national surveillance and facilitates more complete national reporting of human WNV infections to CDC's ArboNET program (*24*).

Our estimate of an average 6.9-day period of viremia detectable by minipool-NAT correlates well with the duration of viremia that was documented after intentional WNV inoculation of human cancer patients in the 1950s (*25*). In those studies, the duration of viremia (detected by intracerebral inoculation in mice, which is less sensitive than minipool-NAT) correlated with underlying disease severity and averaged 6.2 days in a subset of relatively healthy patients.

Our results have limitations. We assumed that WNV incidence in blood donors reflects incidence in the general population. Blood donors differ from the general population with respect to age; however, serologic surveys indicate that age is not associated with the likelihood of WNV infection acquisition but is associated with severity of disease (*1,2,6,7*). Some racial, ethnic, and socioeconomic groups are also underrepresented in the blood donor population. Because WNV is a mosquitoborne arbovirus, incidence may vary among these demographic subgroups, which could bias extrapolations based on donor data. Moreover, potential donors with fever or headache are deferred from donation because the combined symptoms may indicate WNV infection; thus, blood donor screening data would underestimate infection incidence in the general population. However, we believe an underestimate is unlikely since the primary viremia phase of infection detected by minipool-NAT tends to precede development of WNV-related symptoms (*1,10,26*).

Although projections of seasonal incidence estimates based on donor data have limitations, they represent a source of data independent from national disease reporting. Completeness of reporting of WNV neuroinvasive cases to ArboNET is unknown and likely varies among states. The ratio of total infections to neuroinvasive cases is also not precisely known, thus adding uncertainty to incidence data extrapolated from such cases. Using blood donor screening data, we project that ≈256 people are infected with WNV for each person in whom neuroinva-

Table. Estimated WNV seasonal incidence and related measures of WNV infection for each state and entire United States, 2003*

State	No. MP-NAT–positive donations, 4-mo period	No. donations, 4-mo period	Estimated WNV seasonal incidence, % (95% CI)	Midyear population estimate†	Estimated no. infections	Neuroinvasive cases reported to CDC‡
Alabama	3	48,044	0.12 (0.00–0.27)	4,500,752	5,336	25
Alaska	0	8,444	0.00 (0.00–0.00)	648,818	0	0
Arizona	2	86,157	0.04 (0.00–0.10)	5,580,811	2,291	7
Arkansas	1	49,750	0.04 (0.00–0.12)	2,725,714	1,050	23
California	2	394,470	0.01 (0.00–0.02)	35,484,453	3,231	2
Colorado	157	65,739	4.33 (1.83–6.83)	4,550,688	197,028	621
Connecticut	1	52,410	0.03 (0.00–0.10)	3,483,372	1,121	12
Delaware	3	19,853	0.28 (0.00–0.63)	817,491	2,268	12
District of Columbia	0	3,055	0.00 (0.00–0.00)	563,384	0	3
Florida	6	248,198	0.05 (0.00–0.09)	17,019,068	7,677	61
Georgia	6	118,981	0.09 (0.00–0.18)	8,684,715	7,803	27
Hawaii	0	16,981	0.00 (0.00–0.00)	1,257,608	0	0
Idaho	0	23,204	0.00 (0.00–0.00)	1,366,332	0	0
Illinois	15	236,926	0.11 (0.03–0.20)	12,653,544	14,399	30
Indiana	4	111,090	0.06 (0.00–0.14)	6,195,643	3,944	15
Iowa	6	89,649	0.12 (0.00–0.23)	2,944,062	3,476	81
Kansas	70	59,673	2.13 (0.85–3.42)	2,723,507	58,136	89
Kentucky	3	68,674	0.08 (0.00–0.18)	4,117,827	3,298	11
Louisiana	6	56,284	0.19 (0.00–0.38)	4,496,334	8,649	101
Maine	0	24,968	0.00 (0.00–0.00)	1,305,728	0	12
Maryland	7	75,403	0.17 (0.01–0.33)	5,508,909	9,418	49
Massachusetts	0	65,914	0.00 (0.00–0.00)	6,433,422	0	12
Michigan	7	174,776	0.07 (0.01–0.14)	10,079,985	7,122	14
Minnesota	22	114,571	0.35 (0.11–0.59)	5,059,375	17,589	48
Mississippi	6	47,980	0.23 (0.01–0.45)	2,881,281	6,548	34
Missouri	9	124,644	0.13 (0.02–0.24)	5,704,484	7,342	39
Montana	8	19,984	0.76 (0.08–1.43)	917,621	6,956	75
Nebraska	161	61,516	4.87 (2.06–7.68)	1,739,291	84,648	194
Nevada	1	32,651	0.06 (0.00–0.17)	2,241,154	1,267	2
New Hampshire	0	24,595	0.00 (0.00–0.00)	1,287,687	0	2
New Jersey	9	98,008	0.17 (0.02–0.31)	8,638,396	14,269	21
New Mexico	10	25,130	0.73 (0.12–1.34)	1,874,614	13,701	74
New York	10	245,357	0.07 (0.01–0.13)	19,190,115	13,774	57
North Carolina	1	146,807	0.01 (0.00–0.04)	8,407,248	1,087	16
North Dakota	30	13,971	4.14 (1.40–6.88)	633,837	26,264	94
Ohio	13	214,819	0.11 (0.02–0.19)	11,435,798	12,112	84
Oklahoma	23	98,287	0.43 (0.13–0.73)	3,511,532	15,196	56
Oregon	1	72,337	0.03 (0.00–0.08)	3,559,596	966	0
Pennsylvania	14	255,806	0.10 (0.02–0.17)	12,365,455	12,010	145
Rhode Island	0	580	0.00 (0.00–0.00)	1,076,164	0	5
South Carolina	0	70,369	0.00 (0.00–0.00)	4,147,152	0	3
South Dakota	46	20,430	4.02 (1.50–6.54)	764,309	30,716	151
Tennessee	4	88,532	0.08 (0.00–0.17)	5,841,748	4,797	21
Texas	80	309,469	0.48 (0.19–0.77)	22,118,509	106,013	431
Utah	0	35,448	0.00 (0.00–0.00)	2,351,467	0	0
Vermont	0	16,616	0.00 (0.00–0.00)	619,107	0	0
Virginia	1	77,634	0.02 (0.00–0.07)	7,386,330	1,623	19
Washington	0	93,469	0.00 (0.00–0.00)	6,131,445	0	0
West Virginia	1	25,560	0.07 (0.00–0.23)	1,810,354	1,342	1
Wisconsin	4	142,456	0.05 (0.00–0.11)	5,472,299	2,863	7
Wyoming	17	9,904	3.48 (0.94–6.02)	501,242	17,439	92
Total	770	4,585,573	0.25 (0.11–0.39)	290,809,777	735,000 (322,000–1,147,000)§	2,866

*WNV, West Nile virus; MP-NAT, minipool-nucleic acid amplification testing; CI, confidence intervals.
†US Census Bureau (*22*).
‡Source: Centers for Disease Control and Prevention (*23*).
§Confidence interval.

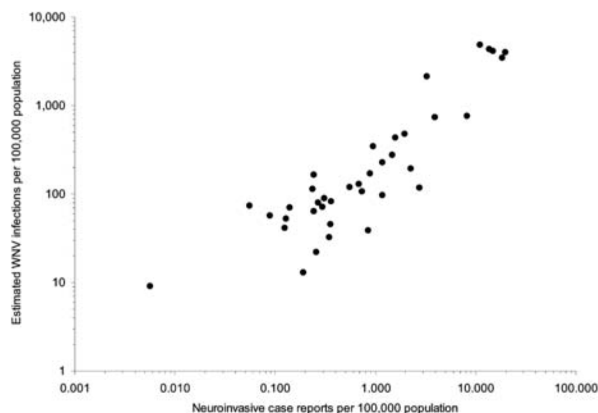

Figure 5. Projected proportion of each state's population infected with West Nile virus versus the proportion of the state's population reporting neuroinvasive disease cases to the Centers for Disease Control and Prevention's ArboNET program. Data are excluded for 13 states: 6 states with neither minipool–nucleic acid amplification testing (MP-NAP) yield nor neuroinvasive cases, 6 states with 2 to 12 neuroinvasive cases but no MP-NAT yield, and 1 state with 1 MP-NAT–positive donor but no reported neuroinvasive cases.

sive disease develops (95% CI 112–401). This ratio is similar to that observed in a serologic survey in Romania, which estimated that 1 in 140–320 infections results in neuroinvasive disease (*6*). Previous estimates of the total number of persons infected in the United States are based on a serologic survey in New York City that indicated that 1 in 140 infections (95% CI 61–217) results in neuroinvasive disease (*2,7*). Although CIs around the New York City estimate and our ratio overlap, the blood donor screening data suggest that previous projections may have underestimated the total number of persons infected. Similar analyses to determine the proportion of infections that result in febrile illness or other clinical manifestation of WNV would be of interest. However, reporting of these illnesses to ArboNet is incomplete and highly variable by state and over time and hence not appropriate for this purpose. Follow-up studies of viremic donors have demonstrated that febrile syndromes develop in 20% to 30% of patients (*26*), consistent with reports from other studies (*1–3*).

Our approach of using NAT yield data to project WNV infections has advantages over serologic strategies. Performing large-scale, community-based serologic surveys to estimate infection incidence is prohibitively expensive, is subject to participation bias, and can be biased by previous exposures to WNV or infections by other flaviviruses that cross-react on WNV IgM and IgG assays (*9,27–31*). Given the extent of recent WNV spread in the United States, interpretation of future serologic surveys will require determination of baseline prevalence before each epidemic year, evaluation of serial samples throughout the epidemic to accurately estimate infection incidence, or both.

In conclusion, our study demonstrates that in addition to preventing many transfusion-transmitted WNV infections, routine donor NAT screening has valuable public health applications, both as an early indicator of human epidemic activity regionally and as a surveillance tool to help monitor national infection incidence. In addition, this study highlights the value of establishing a national system for compiling blood donor data, which would enable ongoing and timely surveillance of WNV and other established and emerging infectious diseases.

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Dr Busch is director of Blood Systems Research Institute and vice-president for research and scientific programs for Blood Systems, a national network of blood centers and donor testing laboratories. He is also a professor of laboratory medicine at the University of California, San Francisco. His major research interests include the epidemiology, natural history, pathogenesis, and laboratory evaluation of transfusion-associated viral infections; and immunologic consequences of allogeneic transfusion, including development of microchimerism and tolerance.

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Aspergillus ustus Infections among Transplant Recipients

Anil A. Panackal,*† Alexander Imhof,*‡ Edward W. Hanley,* and Kieren A. Marr*†

Aspergillus ustus is a mold that rarely infects humans; only 15 systemic cases have been reported. We report the first outbreak of invasive infection caused by *A. ustus* among hematopoietic stem cell transplant (HSCT) recipients. Six patients with infections were identified; 3 infections each occurred in both 2001 and 2003. Molecular typing by using randomly amplified polymorphic DNA (RAPD) and antifungal drug susceptibility testing were performed on clinical and environmental isolates recovered from our hospital from 1999 to 2003. The highest overall attack rate in HSCT patients was 1.6%. The overall death rate was 50%, and death occurred within 8 days after diagnostic culture collection. Clinical isolates exhibited decreased susceptibility to antifungal drugs, especially azoles. RAPD and phylogenetic analysis showed genetic similarity between isolates from different patients. Based on the clustering of cases in space and time and molecular data, common-source acquisition of this unusual drug-resistant species is possible.

Invasive aspergillosis (IA) has become a devastating opportunistic fungal infection among immunocompromised hosts, with a 357% increase in death rates reported in the United States from 1980 to 1997 (*1*). The most common cause of IA is *Aspergillus fumigatus* (*2*). However, in recent years, IA has been increasingly caused by non-*fumigatus Aspergillus* species. For example, at the Fred Hutchinson Cancer Research Center in Seattle, the proportion of infections caused by non-*fumigatus Aspergillus* species increased during the latter 1990s. Most of these infections were caused by *A. flavus*, *A. nidulans*, *A. terreus*, and *A. niger* (*3*).

Aspergillus ustus is a group of filamentous hyalohyphomycetes consisting of 5 species: *A. ustus*, *A. puniceus, A. panamensis, A. conjunctus,* and *A. deflectus*. Members of this group are rare human pathogens; only 15 cases of systemic infection have been reported in the literature since 1970, and more than half of these occurred in the past 10 years (online Appendix Table, available at http://www.cdc.gov/ncidod/EID/vol12no03/05-0670.htm#table_app) (*4–17*). Infections caused by *A. ustus* may be of particular concern, as the organisms exhibit low susceptibility to multiple antifungal drugs, and outcomes have been uniformly poor (Appendix Table). Recognition of invasive infections that occurred in 2 clusters of hematopoietic stem cell transplant (HSCT) recipients in our institution prompted us to perform a more thorough clinical investigation and environmental sampling to identify potential sources of acquisition.

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Methods

Case Identification and Environmental Surveillance

Recognition of time-clustered cases in 2003 prompted us to do this retrospective study and epidemiologic investigation. Cases of infection caused by *A. ustus* were identified by review of microbiology and infection control records available from 1993 to 2003. Charts were reviewed for clinical data (demographics, underlying disease, transplantation characteristics, antifungal therapies, radiographic and laboratory studies, and outcome). Cases were classified as proven, probable, or possible according to consensus criteria published by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (*18*). The Fred Hutchinson Cancer Research Center institutional review board approved this study.

The hospital is a large tertiary care facility that houses patients with HSCT on the top 2 floors (the northeast wings of the seventh and eighth floors). A spot map depicting case-patient location and timeline relating location to

time of diagnosis was created. Information on timing of construction activities and airflow information was obtained from hospital engineering and infection control personnel. An attack rate was estimated among the potentially exposed HSCT patients using as the denominator the number of patients who were admitted for HSCT from July through October 2001 and March through September 2003, the at-risk periods when cases occurred.

Environmental Sampling

Based on the spot map, environmental air sampling of patient hospital rooms was performed, and environmental isolates were obtained. An air particle sampler (SAS Super 100, PBI International, Milan, Italy) was used to collect ambient "dust" to the 0.3-µm size. Samples (0.5 m^3) were cultured on inhibitory mold agar plates (Remel IMA plates, Lenexa, KS, USA). Organisms were identified to the species level by using standard morphologic criteria for *A. ustus*. Isolates were stored at –70°C.

Molecular Typing

Molecular typing of *A. ustus* clinical and environmental isolates was performed by randomly amplified polymorphic DNA (RAPD) analysis by using *A. ustus* ATCC 1041, NRRL 275, and *Candida parapsilosis* for outgroup comparison (*19*). DNA templates were purified from ≈50 mg cells, resuspended in phosphate-buffered saline (PBS), treated with Lyticase 10 µg/mL (Sigma Chemical Co., St. Louis, MO, USA) for 1 h at 37°C, and then digested with Proteinase K 10 µg/mL (Sigma Chemical Co.). Mixtures were subjected to 3 cycles of freeze-thaw in liquid nitrogen, alternating with vortexing with 0.2 g glass beads. Genomic DNA was isolated with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RAPD reactions were run under conditions optimized for each primer (Table 1) by using a PerkinElmer 9700 thermal cycler (PerkinElmer, Cetus, CT, USA). PCR products underwent electrophoresis in 1.8% agarose gels, were stained with ethidium bromide, and images were obtained by using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA). Only bands that possessed one-tenth the integrated intensity of the 1,650-bp band of the molecular marker (4 ng) (area under the curve [AUC] = 1,132) were defined as positive bands for subsequent band relational analysis. The band patterns from each gel with each primer were analyzed by using tools for population genetics analysis (TFPGA) (unpub. data). Cluster analysis was performed by the unweighted pair group mean with arithmetic average (UPGMA) method (*20*). Bootstrapping was performed with 1,000 tree comparisons with averages by using TFPGA. Band patterns of >95% similarity were classified as identical.

Antifungal Drug Susceptibility Testing

Antifungal drug susceptibility testing of *A. ustus* isolates was performed by using a microbroth dilution assay, as described by the National Committee for Clinical Laboratory Standards or the filamentous fungi (M38-A) for itraconazole (Janssen, Titusville, NJ, USA), voriconazole (Pfizer, New York, NY, USA), and amphotericin B (Bristol-Myers Squibb Co., Princeton, NJ, USA) (*21*). Susceptibility (minimal effective concentration) to caspofungin (Merck Research Laboratories, Rahway, NJ, USA) was determined by using a microbroth dilution assay in antibiotic 3 (AM3) media, as described previously (*22*).

Results

Outbreak Cases

We identified 2 clusters of *A. ustus* infection among HSCT recipients in our hospital during the study. The first occurred from July to October 2001 (3 probable lung infections: patients 1, 2, and 3). The second occurred from March to September 2003 (1 proven skin infection [likely disseminated from lung] and 2 probable lung infections: patients 4, 5, and 6) (Appendix Table); 1 lung transplant recipient was colonized with *A. ustus* while in the hospital (data not shown).

The median age of patients was 59 (range 29–63) years; 5 (83.3%) were male; median neutropenia duration 15 (range 4–22) days; 5 (83.3%) patients had graft-versus-host disease that required therapy; 5 (83.3%) patients had received mold-active antifungal drugs prophylactically (itraconazole, n = 4) or for a prior diagnosis (voriconazole, n = 1). The median time of diagnosis after transplantation

Table 1. Conditions for *Aspergillus ustus* DNA amplification

	Primers				
	Ustus 1	R151	RPO2	OPA10	OPA20
Primer sequence	5′-GTA TTG CCC T-3′	5′-GCT GTA GTG T-3′	5′-GCG ATC CCC A-3′	5′-GTG ATC GCA G-3′	5′-GTT GCG ATC C-3′
Primer concentration	0.8 pmol/L	1.0 pmol/L	1.0 pmol/L	0.4 pmol/L	1.0 pmol/L
$MgCl_2$	1.8 mmol/L	2.2 mmol/L	3.0 mmol/L	1.8 mmol/L	2.0 mmol/L
Template concentration	0.025 ng/50µL	0.5 ng/50µL	0.012 ng/50µL	0.03 ng/50µL	0.1 ng/50µL
Annealing temperature	32°C	32°C	34°C	32°C	32°C
Annealing time	1.5 min	1.5 min	1.5 min	1.5 min	1.5 min

was 222 (range 60–1,295) days (n = 5). Three (50%) of the 6 patients died, all within 8 days of diagnostic culture collection.

Epidemiologic Investigation

Estimating that 382 patients were admitted for HSCT during the at-risk period, the highest overall attack rate was 1.6%, which is above the baseline rate of infection with *A. ustus* at our institution (0%). No changes in laboratory processing or mold identification methods occurred during the study. Of note, construction of a new surgery pavilion occurred outside our hospital building beginning in July 2001 and ending in December 2003. Airflow to hospital rooms in which the patients resided passed through multiple filters (blanket filter, pre-filter, 95% filter, HEPA filter).

The spot map and time line showed that cases clustered mainly along 2 corridors on 2 floors, 1 directly above the other, around the time of diagnosis. In the 2001 and 2003 outbreaks, all case-patients resided in the same or adjacent rooms before diagnosis (Figure 1).

Environmental air sampling performed 2 months after the last case occurred in 2003 found no *A. ustus* isolates in the rooms of HSCT patients. One environmental *A. ustus* isolate was obtained from the carpeted floor of the hall near the room in which the colonized lung transplant recipient resided. The same bronchoscope was used to evaluate each patient; however, it was cleaned after each examination. Also, several patients who were not found to have *A. ustus* on bronchoalveolar lavage underwent bronchoscopic examination before case-patients, suggesting that cross-contamination was unlikely.

Analyses of Isolates

Eleven *A. ustus* isolates were available for analysis. One patient (patient 3) did not have a viable isolate stored, and 1 patient (patient 5) had 3 isolates recovered during the course of infection. A total of 73 bands were resolved from the 11 *A. ustus* isolates (Figure 2). The isolates recovered from the 5 HSCT were genetically similar. Three isolates from patient 5 were genetically most similar to the isolate from patient 2. At the time of his diagnosis and death, patient 2 resided in a room directly adjacent to and above the room of patient 5, albeit 2 years earlier (Figure 1). Similarly, the isolate from patient 1 was genetically most similar to that of patient 4; patients 1 and 4 resided in adjacent rooms, also separated by a period of 2 years. Of note, the lung transplant patient appeared to be colonized with a strain of *A. ustus* that was genetically as distant from the patient isolates as the wild-type ATCC strain. Antifungal drug susceptibility testing of clinical isolates demonstrated relatively high MICs to all antifungal drugs tested (Table 2).

Discussion

We report the first outbreak of disease caused by an unusual fungal pathogen, *A. ustus*, a mold that has rarely caused invasive disease in humans. This observation is

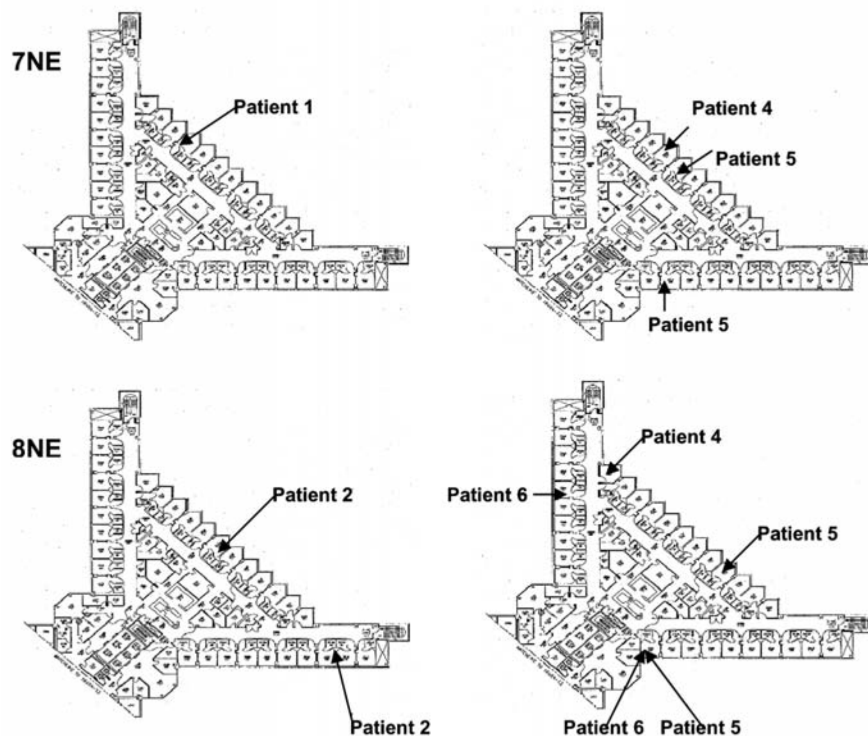

Figure 1. Spot map illustrating case-patient location on the northwestern wing of the eighth floor (8NE) and the seventh floor (7NE) from the 2001 (left panel) and 2003 outbreaks (right panel) at the time of case diagnosis. Patient 3 was in the outpatient clinic at the time of diagnosis and is, therefore, not marked on this inpatient spot map. Patient 5 and 6 resided in the same room at different times. Patients 2, 4, 5, and 6 were moved to a variety of rooms around the time of diagnosis as indicated by their location in multiple rooms.

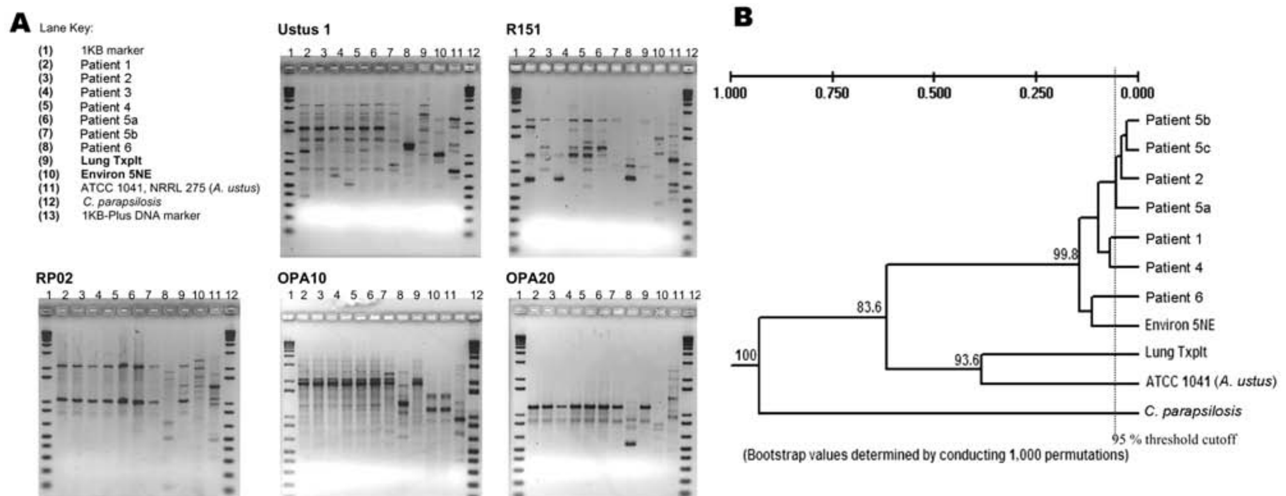

Figure 2. Molecular typing of *Aspergillus ustus* isolates by using random amplification of polymorphic DNA. The isolate from patient 3 was not viable on subculturing and, as such, was not available for molecular analysis. Gel images (A) and composite dendrogram (B) are shown.

important, given the possibility of common-source acquisition of a potentially antifungal drug–resistant organism.

All members of the *A. ustus* complex have similar shape with subtle differences. Macroscopically, the colonies appear drab olive to dull brown or gray and wooly with occasional dark purple or yellow exudates. Microscopically, the conidia are large (3.0–4.5 μm) and are rough-walled. Elongate and irregular-shaped Hülle cells that are resistant to desiccation may also be produced (*23*). *A. ustus* is toxigenic and produced several mycotoxins such as austdiol, austin, austocystin A, and sterigmatocystin (*24–27*). Although these toxins may be medically important, the quantities of toxin produced in the environment may not be significant (*28,29*). The spectrum of disease reported due to *A. ustus* includes onychomycosis, otitis media, primary cutaneous infection, endocarditis, pneumonia, and disseminated infection, the latter cases occurring largely among immunocompromised hosts such as HSCT recipients. All previously reported cases occurred sporadically in diverse medical centers (Appendix Table). Many reported cases have been either primary cutaneous disease or disseminated infection, however, we cannot draw firm conclusions regarding the types of infections this organism causes because of the high likelihood of reporting bias. The relative pathogenicity of this *Aspergillus* species has not been well studied.

In the 6 HSCT patients described in this article, infection developed late after HSCT, with a high proportion of deaths (*30,31*). These patients also possessed classic risk factors for IA in that most had graft-versus-host disease that required corticosteroid and other immunosuppressive therapy (*30*). Overall death rates of patients with *A. ustus* infection was high in this cohort, as in previous cases (*4–15*). Whether death was attributable to the fungal infection, coinfections, or underlying diseases is unclear.

A common source for the *A. ustus* infections appears possible, since case-patients clustered in space and time, and a high degree of genetic similarity was noted between isolates from case-patients. Since these patients resided in rooms within close proximity, common source acquisition (e.g., air, water, or surface) is credible. Common source acquisition may not be precluded by case isolate separation in time as *Aspergillus* conidia are resistant to harsh conditions, surviving in the environment for many years in dormant phase (*32*). However, the environmental niche of this fungus is not known. Patients were in and out of the hospital after transplantation, so infection could have been acquired in the environment. We also cannot rule out the possibility that other clinical factors (e.g., changes in hosts or antifungal drug administration) selected for specific *A. ustus* isolates in the patients.

The results of molecular analyses suggest genetic similarity of isolates recovered from patients. Although discriminatory power of RAPD analyses has some limitations (*33*), the composite analysis demonstrated large separations between patient isolates and the control ATCC strain. Our study is limited by the lack of local environmental *A. ustus* isolates available for genetic comparison. Although the clinical isolates appear different from the ATCC strain, the genetic similarity of case strains may represent a strain common to our local environment. Also, these analyses are limited by our lack of knowledge concerning *A. ustus*'s modes of reproduction. Specifically, genomic rearrangement with recombination, which has been postulated to occur in several species of *Aspergillus*, may increase the variation observed between related strains (*34*).

Table 2. Antifungal drug susceptibility testing of *Aspergillus ustus* isolates

Isolate	Amphotericin B*	Voriconazole*	Itraconazole*	Caspofungin†
Patient 1	2.0	8.0	4.0	2.0
Patient 2	2.0	4.0	2.0	2.0
Patient 4	2.0	8.0	4.0	4.0
Patient 5a	1.0	4.0	2.0	8.0
Patient 5b	1.0	8.0	8.0	4.0
Patient 5c	2.0	8.0	4.0	2.0
Patient 6	2.0	8.0	8.0	2.0
Lung transplant	2.0	4.0	1.0	2.0
Environment	4.0	8.0	4.0	2.0

*MIC_{100}, µg/mL.
†Minimum effective concentration, µg/mL.

Our investigation was limited by constraints in conducting retrospective analyses. More timely environmental sampling may have captured more environmental *A. ustus* isolates (*32,35*). For example, swabbing of dust-ridden surfaces may have indicated the underlying air quality in terms of fungal spores in the preceding months when infection may have occurred. In the absence of substantial air disturbances, *A. ustus* spores would be more likely to quickly settle in such areas, given their large size and relatively decreased buoyancy. Construction, a well-known environmental risk factor for IA (*36*), was ongoing outside the hospital during the time of these outbreaks. We cannot comment on the role of water as a source of infection, which has been reported in multiple hospitals (*32,35*). Determining the source of infection is further complicated in that a combination of inoculum effect and underlying host immunosuppression make calculating the incubation period problematic. Thus, the source of *A. ustus* infection among our patients, and whether the infections were nosocomial or community acquired, remains unknown.

These outbreaks of *A. ustus* infections may be of infection control importance, as the clinical isolates exhibited low susceptibilities to multiple antifungal drugs, as was reported previously (*12,17*). Although we do not know breakpoints of *A. fumigatus* resistance, results of prior studies suggest that infection with organisms requiring high MICs of amphotericin or itraconazole is associated with poor clinical outcomes (*37,38*). Most of our patients received mold-active azole drugs before diagnosis as either prophylaxis or therapy for a previous infection with *A. fumigatus*. Similarly, *A. ustus* was recently reported to cause "breakthrough" infection during administration of voriconazole and caspofungin (*17*). Drug exposure may select for colonization or infection with resistant isolates or facilitate acquired resistance within a colonizing strain. The latter may occur in *A. fumigatus* isolates exposed to azole antifungal agents (*39,40*). In this cohort, several patients who received the combination regimen of voriconazole and caspofungin had *A. ustus* infection resolve; whether this resolution was due to drug synergy in treating relatively resistant organisms is worthy of further consideration.

A. ustus is rare; however, it may be emerging as a cause of systemic disease among immunocompromised hosts in the appropriate setting. A combination of factors, including severity of underlying host immunosuppression and common source acquisition, likely played a role in the reported outbreaks. Active laboratory, environmental, and clinical-based surveillance for *A. ustus* has been implemented at our hospital based on the results of this investigation; no additional isolates have been identified subsequently. Such intensive monitoring may show similar outbreaks in other facilities. This study also emphasizes the importance of establishing microbial diagnoses to the species level; information obtained is important for infection control and, possibly, to guide antifungal therapies. More studies will be necessary to determine the clinical consequence of antifungal resistance in *A. ustus* isolates.

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Clostridium difficile Infection in Patients Discharged from US Short-stay Hospitals, 1996–2003[1]

L. Clifford McDonald,* Maria Owings,* and Daniel B. Jernigan*

US hospital discharges for which *Clostridium difficile*–associated disease (CDAD) was listed as any diagnosis doubled from 82,000 (95% confidence interval [CI] 71,000–94,000) or 31/100,000 population in 1996 to 178,000 (95% CI 151,000–205,000) or 61/100,000 in 2003; this increase was significant between 2000 and 2003 (slope of linear trend 9.48; 95% CI 6.16–12.80, p = 0.01). The overall rate during this period was severalfold higher in persons >65 years of age (228/100,000) than in the age group with the next highest rate, 45–64 years (40/100,000; p≤0.001). CDAD appears to be increasing rapidly in the United States and is disproportionately affecting older persons. Clinicians should be aware of the increasing risk for CDAD and make efforts to control transmission of *C. difficile* and prevent disease.

Clostridium difficile is an anaerobic, spore-forming bacillus that produces 2 important exotoxins: toxin A, an enterotoxin, and toxin B, which is primarily a cytotoxin (*1*). *C. difficile* is the most commonly recognized cause of antimicrobial drug–associated diarrhea. Although *C. difficile*–associated disease (CDAD) is usually localized to the large bowel, where it manifests as diarrhea and pseudomembranous colitis, disease may progress to toxic megacolon, sepsis with or without intestinal perforation, and death (*2*) CDAD is increasingly recognized among residents of long-term care facilities (*3*) and even among persons living in the community (*4*); however, it most commonly affects patients in short-stay hospitals, where epidemic strains of *C. difficile* may be transmitted extensively both within and between facilities (*5,6*). Moreover, substantial excess healthcare costs and excess hospital days are associated with CDAD among short-stay hospital patients (*7,8*).

Several reports from individual hospitals (*9–11*) and a recent report primarily from the intensive care unit component of the National Nosocomial Infection Surveillance System (NNIS) (*12*) suggest that the incidence of CDAD may be increasing. However, only 90 to 340 hospitals contributed to NNIS during the study period, and these hospitals do not represent a probability sample of all US hospitals. Therefore, we analyzed national hospital discharge data to determine 1) the scope and magnitude of CDAD in US short-stay hospitals, 2) whether the rate of CDAD was indeed increasing, and if so, 3) the epidemiologic factors associated with such an increase.

Methods

The National Hospital Discharge Survey (NHDS) is conducted annually by the National Center for Health Statistics, Centers for Disease Control and Prevention (CDC), and consists of diagnosis and demographic data collected from a national probability sample of patient discharge records (*13*). At least 90% of a panel of 500 hospitals participate, from which over 300,000 discharge records are sampled each year. Based upon the analysis weight applied to each record of this ≈1% sample, national estimates were made regarding the number and character of all nonfederal, short-stay hospital discharges. NHDS data were used to determine the number of discharges with the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) code (008.45) specific for "intestinal infection due to *Clostridium difficile*" listed as a discharge diagnosis. This code was introduced in 1993 and is the only ICD-9-CM code specific for CDAD.

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National estimates of both the absolute number of discharges with CDAD listed as a discharge diagnosis, the proportions of discharges with CDAD, and the rates per 100,000 US population were determined for the years 1996–2003. Standard errors for all statistics were calculated by using SUDAAN version 7.0 (Research Triangle Institute, Research Triangle Park, NC, USA), which takes into account the complex sample design of the NHDS. A description of the software and the approach it uses have been published (*14*). Data were stratified according to whether CDAD was listed as the first-listed vs. any-listed diagnosis, patient sex, patient age, and hospital geographic region (according to US census regions), bed size, and ownership type (i.e., proprietary, state and local government, or nonprofit). The NHDS collects data on up to 7 diagnoses for each discharged patient sampled. The first-listed diagnosis is the principal diagnosis if it were specified as such on the medical record or the face sheet of the discharge summary of the patient. If the principal diagnosis is not specified, then diagnoses are listed in the order they are given. The principal diagnosis is the condition established after study as chiefly responsible for the hospitalization. Therefore, estimates for the first-listed diagnosis of CDAD refer to patients for whom CDAD is most likely the primary reason for the hospital admission. Any listed diagnosis estimates reflect discharged patients for whom CDAD was either a primary or a secondary diagnosis, including those patients who may have contracted CDAD during the current hospitalization, as well as those who acquired it by other means.

Rates of discharges with CDAD as any diagnosis over the entire study period (i.e., 1996–2003) were compared by various demographic factors using the 2-tailed Z-test based on standard errors obtained from SUDAAN. Tests of linear trend over time in rates, percentages, and numbers were performed by using a weighted least-squares regression method; p values were determined from the *t* statistic by using degrees of freedom equal to 1 less than the number of years over which trends were examined (*15*). Because the trend in overall and stratified rates changed so remarkably at the midpoint of the study period, separate trend analyses were performed for 1996–1999 and 2000–2003. Although these separate trend analysis were performed for all stratified rates presented, only the most pertinent negative findings are presented, along with all significant trend results (i.e., $p<0.05$).

Results

From 1996 through 2003, an estimated 264,000 (95% confidence interval [CI] 232,000–296,000) and 978,000 (95% CI 869,000–1,087,000) discharges for which CDAD was listed as either first or as any diagnosis, respectively. No significant trend was found in the numbers or rates of discharges with CDAD listed as either the first or any diagnosis from 1996 to 1999. In contrast, the number of discharges for which CDAD was listed as the first diagnosis increased from 25,000 (95% CI 19,000–31,000) in 2000 to 54,000 (95% CI 41,000– 67,000) in 2003 (slope expressed as the average change in value per annum [*b*] = 9,000, 95% CI 5,000–13,000, $p<0.001$). Annual increases in the point estimates of these discharges over the precedent year were 43%, 18%, and 27% in 2001, 2002, and 2003, respectively, although the single year increases in 2002 and 2003 were not significant. Meanwhile, the number of discharges for which CDAD was listed as any diagnosis nearly doubled from 98,000 (95% CI 84,000–112,000) in 1996 to 178,000 (95% CI 151,000–205,000) in 2003 (b = 28,000, 95% CI 19,000–38,000, $p<0.001$). Annual increases in the point estimates of these discharges over the preceding year were 43%, 21%, and 5% in 2001, 2002, and 2003, respectively; again, the only significant single-year increase was in 2001. The estimated population-based rates of discharges with either a first-listed or any diagnosis of CDAD during this period are presented in Figure 1, along with the 95 CIs for these estimates. The upward trends in rates of CDAD both as first-listed (b = 3.1, 95% CI 1.70–4.48, p = 0.008) and as any (b = 9.48, 95% CI 6.16–12.80, p = 0.01) discharge diagnosis were significant between 2000 and 2003.

Based upon the estimated 250 million discharges from 1996 to 2003, the proportions of discharged patients with CDAD listed as first or any diagnosis were 0.10% (95% CI 0.092–0.11%) and 0.38% (95% CI 0.35%–0.41%), respectively. Both of these rates increased in a fashion similar to that of population-based rates shown in Figure 1. For CDAD as the first-listed discharge diagnosis, a significant upward trend was evident, from 0.08% in 2000 to 0.16% in 2003 (b = 0.024, 95% CI 0.014–0.0035, p = 0.02). For CDAD as any discharge diagnosis, a significant upward trend occurred from 0.27% in 2000 to 0.51% in 2003

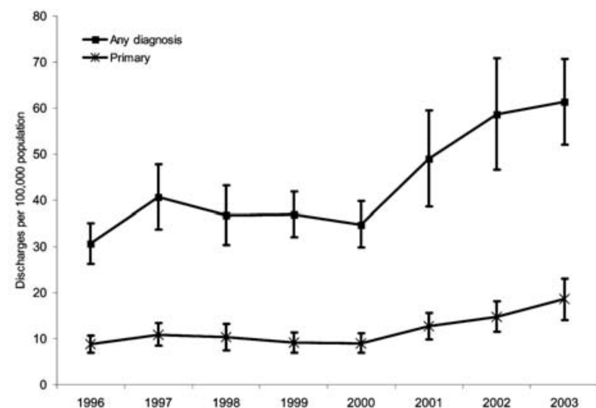

Figure 1. National estimates of US short-stay hospital discharges with *Clostridium difficile* listed as primary or as any diagnosis. Isobars represent 95% confidence intervals.

(b = 0.072, 95% CI 0.048–0.095, p = 0.009). Rates of any CDAD discharge diagnosis over the entire study period (1996–2003) are shown stratified by various demographic factors in the Table; overall rates during this period were identical in male and female discharged patients.

However, the overall rate was severalfold higher in persons ≥65 years of age than in persons ages 45–64 years, and the rate in persons ages 45–64 years was in turn higher than rates in persons ages 15–44 years and persons ages <15 years (Table). In addition, significant increasing trends were found between 2000 and 2003 in both of the older age groups; however, the slope of the increase was several fold greater in those ages ≥65 years (b = 58.1, 95% CI 36.5–79.7, p = 0.01) than those ages 45–64 years (b = 7.9, 95% CI 4.0–11.7, p = 0.03) (Figure 2).

Regional rates of CDAD as any discharge diagnosis are shown in Figure 3. Although rates appeared to increase in each US region from 2000 to 2003, a significant linear trend was found only for the Midwest (b = 13.1, 95% CI 5.4–20.8, p = 0.04) and South (b = 7.9, 95% CI 3.4–12.3, p = 0.04). Overall, during 1996–2003, the rate of CDAD as any discharge diagnosis was higher in the Northeast than in the Midwest, South, and West (Table).

The proportions of discharges, including CDAD as any diagnosis, stratified according to hospital size, are shown in Figure 4. Although rates appeared to increase in each group, from 2000 to 2003, a significant linear trend was found only for hospitals with 100 to 299 beds (b = 0.066, 95% CI 0.027–0.10, p = 0.04) and >300 beds (b = 0.070, 95% CI 0.04–0.10, p = 0.02). Overall rates for 1996–2003 were similar between hospitals with 100–299 beds and hospitals with ≥300 beds, whereas rates in hospitals with <100 beds were significantly lower than rates in either group of larger sized hospitals (Table).

The absolute number of CDAD patients who were transferred to a long-term care facility increased from 20,000 (95% CI 13,000–28,000) in 2000 to 57,000 (95% CI 43,000–71,000) (b = 13,000, 95% CI 8,000–17,000, p = 0.01). CDAD discharges also accounted for an increasing proportion of all patients who were transferred to long-term care facilities during this period, rising from 0.78% (95% CI 0.52–1.04) of long-term care transfers in 2000 to 1.87% (95% CI 1.48–2.26) in 2003 (b = 0.37, 95% CI 0.23–0.52, p = 0.01). Although the point estimate of CDAD inpatients who died before discharge increased from 8,000 (95% CI 5,000–12,000) in 2000 to 15,000 (95% CI 10,000–20,000 in 2003, this trend was not significant (b = 1,910, 95% CI 100–3,700, p = 0.1). Moreover, the proportion of deaths among CDAD discharges did not change significantly: 8.52% (95% CI 5.40–11.64) in 2000 to 8.39% (95% CI 6.09–10.69) in 2003 (b = 0.25, 95% CI 0.90–1.40, p = 0.70).

Discussion

We found both the number and rate of US short-stay hospital discharges with a diagnosis of CDAD increased from 2000 through 2003. The overall rate and increase in the number of discharges were most prominent among patients ≥65 years of age. Although the overall rate was highest in the Northeast, a significant linear increase was found only in the Midwest and South. Rates increased similarly in middle- and large-sized hospitals according to number of beds, and overall rates were similar in these 2 strata; in contrast, small hospitals had a lower overall rate and did not experience a significant increase.

Our study had several limitations. We analyzed only hospital discharge data and the sensitivity and specificity of hospital discharge coding for CDAD are largely

Table. Overall rates of any listed CDAD discharge diagnosis by various demographic factors, 1996–2003*

Demographic factor	Point estimate of rate†	95% confidence interval†	p value
Sex			
Male	0.38%	0.34%–0.42%	
Female	0.38%	0.34%–0.42%	NS
Age group (y)			
≥65	228	200–256	
45–64	40	34–45	<0.001
15–45	11	10–13	<0.001
<15	9	5–9	<0.001
Geographic region			
Northeast	68	56–79	
Midwest	49	36–61	0.03
South	36	27–45	<0.001
West	31	26–37	<0.001
Hospital size by number of beds			
<100	0.30%	0.23%–0.36%	
100–200	0.42%	0.37%–0.47%	0.004
≥300	0.38%	0.35%–0.40%	0.03

*CDAD, *Clostridium difficile*–associated disease; NS, not significant.
†Per 100,000 population unless otherwise indicated as the proportion (%) of hospital discharges.

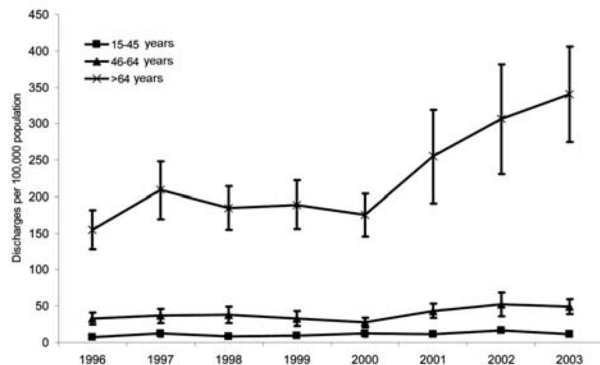

Figure 2. Rates of US short-stay hospital discharges with *Clostridium difficile* listed as any diagnosis, by age. Isobars represent 95% confidence intervals. Because of low rates and the resulting uncertainty of yearly rate estimates, data for patients <15 years of age are not included.

unknown (*16,17*). Although ICD-9-CM code for "an intestinal infection with *C. difficile*" contains the organism name, the code may be used on the basis of the clinical suspicion of CDAD alone, in some instances without a positive laboratory test result. In a single-institution study, when the number of positive *C. difficile* laboratory results were compared to the number of ICD-9-CM coded diagnoses, ICD-9-CM diagnoses overestimated by 32% the number of cases predicted by positive laboratory results (*16*). However, a month-to-month correlation was found between cases detected by a positive *C. difficile* laboratory test result and ICD-9-CM coded diagnosis; when patient medical records of these disparate cases (i.e., agreement with ICD-9-CM code and negative for *C. difficile* laboratory results or not tested) were reviewed in detail, most had a history of CDAD during a previous admission or had a *C. difficile* laboratory test ordered that either had a negative result or was cancelled before specimen collection. Another recent comparison of laboratory and ICD-9-CM data from all US Veterans Affairs hospitals demonstrated that ICD-9-CM coded diagnoses of CDAD underestimated by approximately half the number of CDAD patient discharges determined by positive laboratory results (*17*). These investigators also confirmed that ICD-9-CM coded diagnoses of CDAD correlated with the number of CDAD patient discharges determined by positive laboratory results, both among different hospitals as well as over time within individual hospitals.

Along with the potential insensitivity of coding, limitations involve the sensitivity of commonly used diagnostic tests for CDAD. Many hospital laboratories have migrated away from performing culture for *C. difficile* (sensitive but nonspecific for toxin-producing strains) or tissue culture cytotoxin assays toward the performance of less time-consuming, but generally less sensitive, toxin immunoassays. Data are available on diagnostic methods other than bacterial culture collected as part of proficiency surveys conducted by the College of American Pathologists (CAP) between 1999 and 2003 (*18*). Responses from at least 2,250 North American clinical laboratories surveyed annually suggest that, from 1999 through 2003, $\leq$5% laboratories overall performed tissue culture cytotoxin assays. Although this proportion is small, even if it were much larger, sensitivity could still be an issue as some evidence has shown that even a tissue culture cytotoxin assay performed directly on stool misses a large proportion of patients with diarrhea and low numbers of cytotoxin-producing *C. difficile (19)*.

One possible interpretation of our findings is that the observed increase in CDAD reflects a migration away from immunoassays that detect only toxin A toward use of assays that detect toxin A and B after reports of fatal cases associated with toxin A–negative and toxin B–positive strains (*20*). Indeed, the data from CAP mentioned above indicate such a trend: 78% of laboratories in 1999 performed toxin A immunoassays only and 7% performed toxin A and B immunoassays, whereas by 2003, 55% performed toxin A only immunoassays, and 38% performed toxin A and B immunoassays (*18*). Although this change in testing practices was gradual, even if it had been more sudden, the increased sensitivity of combined toxin A and B immunoassays could not likely explain the observed increase in rates between 2000 and 2003 because toxin A-B–positive strains account for no more than 5% of *C. dif-*

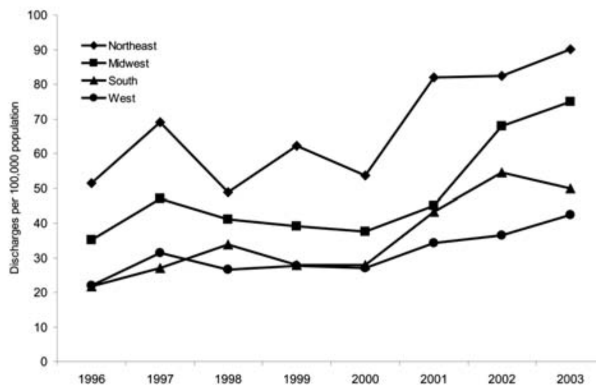

Figure 3. Rates of US short-stay hospital discharges with *Clostridium difficile* listed as any diagnosis, by region. Northeast (Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, Vermont, New Jersey, New York, Pennsylvania), Midwest (Indiana, Illinois, Michigan, Ohio, Wisconsin, Iowa, Kansas, Minnesota, Missouri, Nebraska, North Dakota, South Dakota), South (Delaware, Washington DC, Florida, Georgia, Maryland, North Carolina, South Carolina, Virginia, West Virginia, Alabama, Kentucky, Mississippi, Tennessee, Arkansas, Louisiana, Oklahoma, Texas), and West (Arizona, Colorado, Idaho, New Mexico, Montana, Utah, Nevada, Wyoming, Alaska, California, Oregon, Washington, Hawaii) regions as defined by US Census Bureau.

ficile isolates (21). Nonetheless, to address this issue as well as the possibility that more CDAD cases were being diagnosed due to increased *C. difficile* testing, we analyzed rates of diarrhea using ICD-9-CM codes for nonspecific causes and found no significant decrease. This suggests that the observed increases in CDAD were not simply due to diagnosing more disease among patients with diarrhea of nonspecific causes (data not shown).

Our findings suggest a large impact of excess illness and costs posed by CDAD on the US healthcare system; an impact that may approach or exceed that caused by other more widely recognized nosocomial pathogens. For example, the number of US short-stay hospital discharge diagnoses of CDAD during 2001, 2002, or 2003 exceeded the estimated annual number (120,000) of methicillin-resistant *Staphylococcus aureus* (MRSA) infections for 1999–2000 (*22*). Kyne et al. recently estimated that each case of CDAD in their hospital was associated with $3,699 in excess healthcare costs and 3.6 extra days of hospitalization (*7*). Based upon their hospital's rate of CDAD in 0.7% of discharges, they estimated that the total excess in US healthcare costs attributable to CDAD was likely >$1.1 billion. Even using our lower estimate for the total US cases in 2003 (i.e., 0.51% or 178,000), CDAD can be estimated to have resulted in >$600 million in excess healthcare costs and >600,000 excess hospital days in nonfederal facilities. However, such estimates only account for resource use in short-stay hospitals. Even though some residents infected in long-term care facilities receive their treatment in short-stay facilities, these estimates of excess healthcare costs do not account for the infection control and medication costs incurred within long-term care facilities.

Although our findings are consistent with a recent analysis of CDAD rates in NNIS hospitals (*12*), our results highlight 2 new and unique developments in the epidemiology of this disease. Archibald et al. described a gradual increasing trend between 1987 and 2001 in ICU rates from hospitals with >500 beds and in hospitalwide rates from hospitals with <250 beds (*12*). We found a sharp increase in hospitalwide rates during 2001–2003 following steady rates from 1996 to 2000; this increase was similar in medium and large hospitals. In contrast to early results from NNIS during the spread of MRSA (*23*), when rates were highest in hospitals with the greatest number of beds, we found that overall rates of CDAD were similar in medium and large hospitals.

Several possible explanations may account for the increasing national rates of CDAD. One includes potentially new and evolving patterns of antimicrobial drug use, for example, use of the fluoroquinolones that have recently been implicated in outbreaks of CDAD (*9,24,25*). Another potential contributing factor is the promotion of alcohol-based, waterless, hand sanitizers as the primary means of hand hygiene over soap and water. Because alcohol is not sporicidal, alcohol-based, waterless hand sanitizers may not be as effective as soap and water in removing *C. difficile*; this factor has led to the recommendation that "during outbreaks of CDAD, washing hands with a nonantimicrobial [agent] or antimicrobial soap and water after removing gloves is prudent"(*26*).

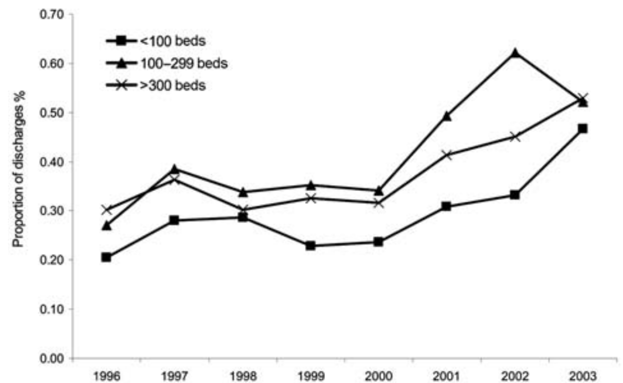

Figure 4. Proportion of US short-stay hospital discharges with *Clostridium difficile* listed as any diagnosis, by hospital size (number of beds).

One important possibility is the emergence of strains of *C. difficile* that are more fit and capable of causing transmission and disease. This emergence would not be unprecedented; in the early 1990s a strain of *C. difficile* that was clindamycin resistant, the so-called "J strain," caused outbreaks in at least 5 geographically diverse hospitals (*5,27*). Indeed, a recent report suggests that an emerging fluoroquinolone-resistant, epidemic strain of *C. difficile* has been responsible for hospital outbreaks in at least 6 US states (Georgia, Illinois, New Jersey, Maine, Oregon, Pennsylvania) since 2001 (*28*). This epidemic strain has continued to spread among additional US states (Connecticut, Florida, Massachusetts, Ohio, Texas), Canada, and Europe (*29*). Another recent report suggests this strain produces 16- and 23-fold more toxins A and B, respectively, than current nonepidemic strains (*30*). Depending on where the earliest outbreaks caused by this epidemic strain were reported, the higher overall rates in the northeastern United States may reflect the early spread of this strain (*9,10,28,29*). If this hypothesis proves correct, it suggests other regions of the United States are likely to observe continued increases as the strain continues to spread geographically.

One striking finding we report is the marked variation in CDAD rates among different age groups, with rates in persons $\geq$65 years of age several fold higher than rates in the next younger age group (45–64 years). Although the importance of advanced age as a risk for CDAD is not a new idea (*1,3*), this is the first report of national CDAD

rates according to age group. Several possible reasons may explain this association between CDAD and age, not the least of which is increased exposure to healthcare facilities (including both acute and long-term facilities) and antimicrobial drugs. In addition, older persons may have decreased host defenses to protect them from CDAD. These conditions include decreased stomach acidity resulting from achlorhydria or a possibly increased use of medications such as histamine-2 receptor blockers or proton-pump inhibitors, medications that are becoming increasingly recognized in association with CDAD (*31,32*). Recent evidence also suggests the importance of a humoral immune response in protecting against CDAD after colonization (*33–37*). Thus, the decreased immune responsiveness commonly observed in older groups may be important in the development of CDAD in patients ≥65 years of age.

Contact precautions are recommended to prevent transmission of *C. difficile* in the healthcare setting (*38*). These consist of placing patients with CDAD in private rooms or cohorting CDAD patients together, using gloves and gowns for all patient contact, and either using disposable patient care equipment or cleaning such equipment between use with different patients. In addition, removing certain potential fomites, such as reusable electronic thermometers, from use in the general hospital patient population is important for controlling outbreaks (*1*). Limited data support enhanced environmental cleaning, especially of heavily contaminated patient care equipment. Clinicians should be aware of the importance of adhering to these precautions for containing transmission of CDAD in healthcare facilities. However, because antimicrobial drug use is the single most important patient risk factor, the clinician's primary responsibility in the control of CDAD lies in the area of judicious antimicrobial drug use (*1*).

In conclusion, the overall scope and magnitude of CDAD are great and may exceed those of other important hospital pathogens (e.g., MRSA), which suggests that *C. difficile* is one of the most common nosocomial pathogens. In addition, the financial costs and patient illness caused by CDAD in US short-stay hospitals appear substantial. Patients ≥65 years of age and those in intermediate- or larger-sized hospitals appear disproportionately affected. Because rates appear to have markedly increased during the first 3 years of this decade, new initiatives in the areas of surveillance, prevention, and control of CDAD are urgently needed. In the meantime, clinicians should be aware of the risk posed by CDAD in their hospitalized patients, remain cognizant of the importance of judicious antimicrobial drug use, and support infection control efforts for CDAD in the healthcare settings where they practice.

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Dr McDonald is a medical epidemiologist in the Division of Healthcare Quality Promotion, National Center for Infectious Diseases, CDC. His main research interests lie in the areas of investigating outbreaks of healthcare-associated infections and understanding and controlling the spread of antimicrobial-resistant pathogens.

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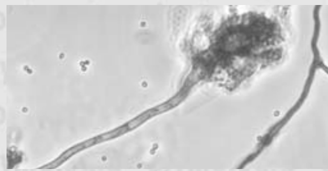
etymologia

Aspergillus

[as″pər-jil′əs]

Genus of filamentous, ubiquitous fungi, commonly isolated from soil, plant debris, and indoor air. *Aspergillus* was first described in 1729 by Pier Antonio Micheli, an Italian priest and biologist who was the first person to attempt the scientific study of fungi. Micheli opposed the idea of "spontaneous generation" by showing that fungal spores grown on a medium would produce the same kind of fungus. The shape of *Aspergillus* reminded him of an aspergillum (from the Latin *aspergere*, "to scatter"), a device used for sprinkling holy water during a liturgical service.



Conidiophore of *Aspergillus fumigatus*. Image courtesy of Libero Ajello, Centers for Disease Control and Prevention.

Courtesy of Davide Borgonovo

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003 and the Illinois Mycological Association, available from http://www.ilmyco.gen.chicago.il.us/

Medication Sales and Syndromic Surveillance, France

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Although syndromic surveillance systems using nonclinical data have been implemented in the United States, the approach has yet to be tested in France. We present the results of the first model based on drug sales that detects the onset of influenza season and forecasts its trend. Using weekly lagged sales of a selected set of medications, we forecast influenzalike illness (ILI) incidence at the national and regional level for 3 epidemic seasons (2000-01, 2001-02, and 2002-03) and validate the model with real-time updating on the fourth (2003-04). For national forecasts 1–3 weeks ahead, the correlation between observed ILI incidence and forecast was 0.85–0.96, an improvement over the current surveillance method in France. Our findings indicate that drug sales are a useful additional tool to syndromic surveillance, a complementary and independent source of information, and a potential improvement for early warning systems for both epidemic and pandemic planning.

Disease surveillance provides essential information for control and response planning. It helps identify changes in incidence and affected groups, thereby providing valuable additional time for public health interventions. Syndromic surveillance aims to use health and health-related data that precede diagnosis or confirmation to identify possible outbreaks, mobilize a rapid response, and thus reduce illness and deaths. This approach is increasingly being explored by public health officials to detect any emerging event (e.g., bioterrorist attacks) and for routine surveillance (*1–6*).

In France, an existing Web-based surveillance system that uses a syndromic approach by collecting weekly office visits to general practitioners provides forecasts of influenza. This approach, based on the method of analogs, produces reasonably sensitive forecasts of annual influenza epidemics (interpandemic influenza) (*7*). However, the method uses past observed patterns of influenzalike-illness (ILI) to forecast future incidence of influenza and may not be able to detect new or unusual public health events, such as the emergence of a pandemic strain of influenza or a bioterrorist attack. For this reason, we investigated other potential data sources associated with ILI that do not rely on past information to forecast incidence and are flexible enough to detect unusual increases in incidence. Here, we evaluate the potential benefit of using a complementary and independent dataset to forecast ILI and eventually to detect influenza epidemics in France. We also compare 2 surveillance methods that use a syndromic approach (one that monitors syndromes defined in clinical terms [ILI] and the other that concerns syndromes defined by using a constellation of drug-specific pharmacy sales indicators). Drug sales have the advantages of providing data on widely used products and of being available in real time. Purchases of drugs could be rapidly relayed to public health authorities, potentially providing lead time for epidemic response planning (*8*).

Materials and Methods

Drug Sales

We used 2 data sources aggregated at the national and regional level. The first database consists of most weekly prescription and over-the-counter (OTC) drug sales, from July 1, 2000, to August 22, 2004, provided by IMS France (http://www.imshealth.com). These data are available in quasi–real time; 7–10 days of lag time are needed for

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quality control and consolidation. The database includes 11,000 pharmacies throughout France (≈50% of all pharmacies) at the regional level (21 regions). The data, consisting of nearly 500 classes of medications, give the number of units dispensed or sold during a certain week for each class of drugs, identified by their codes in the European Pharmaceutical Marketing Research Association Anatomical Therapeutic Chemical (ATC4) classification. In this international classification, drugs are identified by a unique ATC4 code, which corresponds to their primary use. A panel of experts from the World Health Organization Collaborating Center selected 19 classes of medications (Table 1) likely to be prescribed or purchased for ILI. This preselection also avoids the construction of saturated models.

For all years (2001–2004), an aberration in the data for the first week of January was present, likely due to the New Year's holiday. We used the preceding and following weeks to estimate for the week of January 1. Figure 1 shows the temporal trends of sales of 2 of the 19 classes of medications (cephalosporin and expectorants) used in this analysis as an example, as well as the concomitant national ILI incidence. The other classes of drugs included in the analysis follow similar temporal trends (data available on request from the authors).

Table 1. Classes of drugs likely to be prescribed or purchased for influenzalike illnesses

Medication
Cephalosporin*†
Cough suppressant with another medication*†
Cough suppressant with bronchial-pulmonary antimicrobial drug*†
Expectorant*†
Topical nose cream*†
General rhinosinusitis preparation*
Macrolide*
Nasal decongestant*
Nonnarcotic analgesic*
Other antimicrobial agent*
Penicillin*
Rhinocorticoids without antiinflammatory agent*
Tetracycline in association with another medication*
Vitamin C only*
Pharyngeal antiinflammatory decongestant
Antiviral except anti-HIV
Cough suppressant only
Nasal antiinflammatory except corticoids
Vitamin C in association with another medication

*Significant ($p<0.05$) medication classes that were included in the 1-, 2-, and 3-week-ahead national predictive models.
†Medication classes included in >50% of regional predictive models.

ILI Incidence

Data on ILI incidence were obtained from the French Sentinel Network (FSN), which comprises voluntary sentinel general practitioners who update a Web-accessible database with information on communicable diseases including ILI. Weekly national and regional ongoing ILI incidence estimates are published on the Web (http://www.sentiweb.org). ILI is defined by sudden onset of fever of ≥39°C, respiratory symptoms, and myalgia. Epidemic weeks are defined according to a periodic seasonal regression model (based on the concept of excess deaths—here, excess illness—introduced by Serfling [*9*]) that is used routinely in FSN (*10,11*). Epidemic onset is defined as the first week in which the national ILI incidence exceeds a baseline nonepidemic threshold given by the upper limit of the 95% confidence interval of the Serfling model, provided the incidence remains above this threshold for at least 2 consecutive weeks.

Model Construction

We used a Poisson regression model to forecast incidence of ILI based on medication sales. The model allows for overdispersion (when the variance may be larger than the mean in the raw data on ILI incidence and medication sales). The exponential of the estimated Poisson regression coefficients indicates the relative influence of each medication on the incidence of ILI in France. For the explanatory variables (i.e., drug sales data), various time lags were tested (from 0 to 4 weeks). To avoid correlation generated by several lagged values of the same variable (which can bias estimated variance of calculated coefficients), only 1 lagged version of a given explanatory variable was kept. We kept only the time-lagged variable (i.e., sales from 0, 1, 2, 3, or 4 weeks lagged) most correlated to ILI incidence.

Variables were introduced in the model by stepwise selection at the 5% significance level. Sine and cosine terms were included in the model to control for the annual seasonality of ILI incidence. Autoregressive terms (i.e., past terms of the ILI time series) were included if necessary in the final model to eliminate the autocorrelation of the residuals, a common problem in time-series data. The final structure of the model is:

$$\text{Observed incidence of ILI [week } t] = \exp\{\text{intercept} + coeff \times \text{observed incidence of ILI [week } (t - tILI)] + coeff \times \text{sales of drug A[week } (t - tA)] + coeff \times \text{sales of drug B [week } (t - tB)] + \ldots + coeff \times \text{sine}(2\pi t / 52) + \text{coeff} \times \text{cosine}(2\pi t / 52)\},$$

where drug A, drug B, and the like correspond to classes of drugs marked by an asterisk in Table 1, *coeff* denotes respective coefficients of included variables, and *tILI*, *tA*, *tB*, and the like represent respective time-lags of these variables. We constructed 1-, 2- and 3-week-ahead predictive models at national and regional levels on a training dataset corresponding to the period from July 1, 2000, to September 14, 2003, when 3 outbreaks occurred. More details on the predictive models are provided in the online Appendix (available from http://www.cdc.gov/ncidod/EID/vol12no03/05-0573_app.htm).

Model Evaluation

A jackknife-based resampling procedure (*12*), which produces error bounds on the estimate of regression coefficients computed from samples that leave out 1 observation at a time, was used to check the model fit. The models were validated by forecasting the 2003–2004 influenza season (September 15, 2003–August 22, 2004). Models' parameters were reestimated each week with updated data on medication sales and ILI incidence. The predictions of ILI incidence from drug sales were evaluated by Spearman correlation coefficients. The correlation between observed and forecasted incidences was assessed for each forecasting horizon (1, 2, and 3 weeks ahead) for the entire 2003–2004 influenza season (September 15, 2003–August 22, 2004) and for the preepidemic and epidemic weeks (October 6, 2003–January 4, 2004). When regional models were evaluated, the correlation was calculated as the average of the 21 regional correlation coefficients.

We compared the results of the proposed method to the current forecasting approach, the method of analogs, for the national model. The method of analogs, currently employed by FSN, uses weighted sums of vectors selected from historical influenza time series that match current activity to construct forecasted incidences (*7*). All statistical procedures were generated with SAS software, version 8 (SAS Institute, Cary, NC, USA).

Results

National ILI Incidence Forecast

The fitted predictive models for the training dataset for 1, 2, and 3 weeks ahead included 14 of the 19 preselected classes of drugs likely to be prescribed or purchased for ILI (Table 1). The correlation coefficients calculated on the training dataset between observed and model-recalculated ILI incidences were 0.94, 0.92, and 0.91 for 1-, 2-, and 3-week-ahead predictions, respectively ($p<0.001$, Figure 2).

The validation of these models, evaluated first on the entire period from September 15, 2003, to August 22, 2004, and secondly on the preepidemic and epidemic weeks of the 2003–2004 influenza season, provided correlation coefficients of 0.85 to 0.96 (Table 2). The correlation decreased as the time horizon for the forecast increased. The method detects well the beginning of the epidemic but overestimates the epidemic size (data not shown).

The prediction accuracy of our drug sales–based model at a national level was compared with that of the current forecasting method (the method of analogs). As illustrated in Table 2, although the correlation coefficients lie in the same range of values for both methods, they are generally higher with our method.

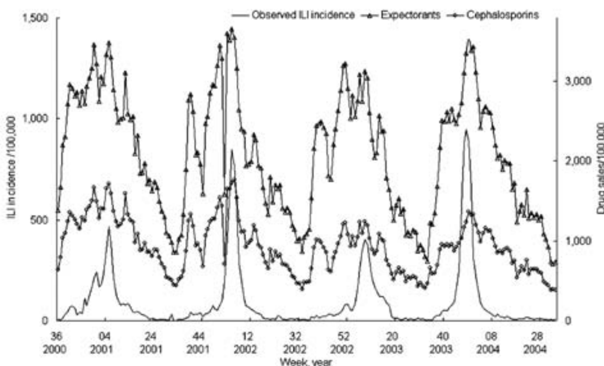

Figure 1. Example of weekly consumption of expectorants and cephalosporins (provided by IMS France) in phase with weekly incidence of influenzalike illness (ILI) (data from French Sentinel Network) per 100,000 population.

Regional ILI Incidence Forecast

At the regional level, 5 classes of medications appeared in at least half of the final selected models (Table 1). These variables are also the most informative in the national model (likelihood test).

The prediction accuracy, defined here as the average correlation coefficient for the 21 regions of France, was 0.54–0.70; it decreased slightly with the forecasting horizon. Compared to the national model, the regional models performed less well. Our regional predictive models gave higher correlation values than the method of analogs for both periods and all forecast horizons except when the ILI incidence was calculated 1 week in advance (Table 2). Forecasted versus observed regional ILI incidences were mapped (Figure 3) for the 6 first weeks of the 2003–2004 influenza epidemic (November 3, 2003–December 14, 2003). Each map of predicted regional ILI incidences was constructed at 1-, 2-, or 3- week horizons. For example, for week 49 of 2003, hereafter designated 2003(49), we provided a 2-week-ahead prediction of ILI incidence, calculated by employing the model using data until the week 2003(47).

Discussion

Our work presents a real-time approach to detect influenza outbreaks and predict trends of ILI incidence 1, 2, or 3 weeks ahead with good reliability. Our method, based on drug sales data, provides similar results as the method that uses report of visits for ILI from sentinel physicians.

The set of drug classes proposed for inclusion in the model was preselected by a panel of experts at the World Health Organization Collaborating Center, based on what they determined to be clinically relevant. Because >500 medication classes are included in the database, we selected a smaller number to avoid overparameterization. This a

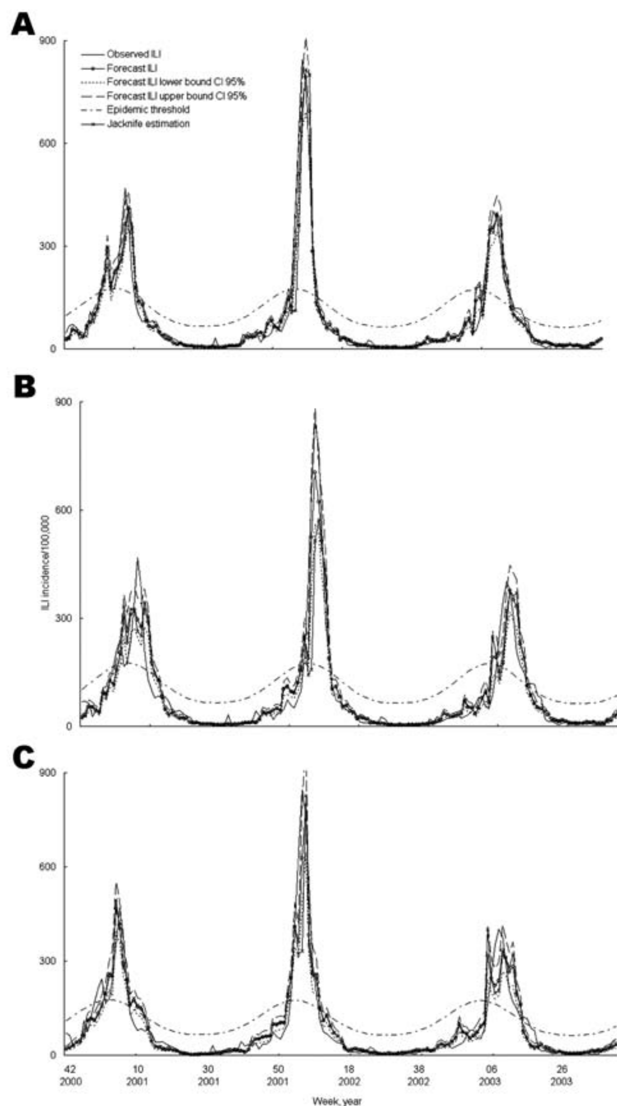

Figure 2. Model construction for national influenzalike illness (ILI) incidence forecasting 1, 2, and 3 weeks ahead for the training dataset (week 36 of year 2000 to week 38 of year 2003) by using a jackknife reestimation procedure (95% confidence intervals [CI] for estimations are given). Forecasted incidence is per 100,000.

priori selection may have influenced our results, in particular application of the model at the regional level. Regional demographic, climatologic, and cultural differences may influence the types of medication prescribed and purchased.

After the stepwise procedure for inclusion in the Poisson regression models, the selected medication groups were both OTC and prescription medications purchased and prescribed for varying degrees of severity of ILI symptoms or complications of influenza. For example, an OTC drug such as vitamin C may be purchased before or at the onset of ILI symptoms since popular beliefs and advertising suggest it may prevent infection or lessen symptom severity (*13*), even in the lack of any evidence or regulatory approval. Cephalosporins, second-line antimicrobial agents, are often used to treat acute bacterial rhinosinusitis, a complication of ILI symptoms present for an extended period (*14*).

At the national level, our forecasting model showed overall good agreement with the observed data on ILI incidence from the FSN surveillance system (Table 2). Over time, the correlation coefficients between observed and forecast ILI time series decreased, although they remained >0.85. The fact that the model was updated on a weekly basis contributed to the overall stability of the method's accuracy. However, the method does not perform as well when used as a tool to quantify the overall epidemic impact (data not shown). Because the main objective of the method is to provide advance warning for onset of the epidemic, this limitation is less important.

At the regional level, the medications included varied from area to area, but 5 drug therapeutic classes were selected for all models (Table 1). This variation may be explained by the fact that while regional similarities exist, different external factors could influence medication consumption. In terms of the forecast accuracy, the correlation coefficients averaging over the 21 regions of France were weaker than those obtained at the national level on the validation dataset (range 0.54–0.70). This may be due to the method itself, which may perform less efficiently at a regional level, or to the quality of the observed regional ILI datasets, since they are obtained from a sample of sentinel physicians. However, the accuracy at the regional level may be sufficient for operational purposes, since the qualitative trend is more relevant than the quantitative evaluation. Using the method at the regional level also provides an additional means to follow the spatial diffusion of the epidemic wave on the basis of a robust and powerful sample of pharmacies distributed all over the country.

We compared our drug sales–based forecasting models to a nonparametric method routinely used by FSN (method of analogs) (*7*). The results obtained appear to be better than those obtained with the method of analogs, but the comparison between the 2 methods is only partial: the method of analogs exploits historical trends to forecast forthcoming ILI events, whereas the regression analysis does not (except for the autoregressive term). In the event of an influenza pandemic or other event not previously observed, our method would be more likely to predict trends that have never been seen in the recent interpandemic past than the method of analogs, which uses a 20-year time series to forecast the future.

As with all forecasting models, the results of this research highlight changes in trends rather than prediction of actual incidence. Only 4 epidemic seasons of data were

Table 2. Forecast accuracy*†

Forecast method of ILI incidence	Forecast horizon (wks)	National level: Weeks 2003(40)–2004(34)	National level: Weeks 2003(41)–2004(01) (preepidemic and epidemic)	Regional level: Weeks 2003(40)–2004(34)	Regional level: Weeks 2003(41)–2004(01) (preepidemic and epidemic)
Using drug-sales data	1	0.93	0.96	0.70	0.64
	2	0.93	0.95	0.70	0.56
	3	0.91	0.85	0.69	0.54
Current method used by FSN	1	0.90	0.96	0.73	0.67
	2	0.93	0.88	0.68	0.53
	3	0.91	0.74	0.65	0.40

*Defined as the correlation coefficient between observed and predicted influenzalike incidences and calculated on the validation dataset. Correlations were computing for 1-, 2-, and 3-week-ahead prediction obtained from medication sales for 2 periods ($p<0.001$). Forecast accuracy was compared with that of the method of analogs. At the regional level, the value in the table was obtained by averaging the correlation coefficient over the 21 regions of France. The number in parentheses after a year refers to that week of that year.
†ILI, influenzalike illness; FSN, French Sentinel Network.

available to both fit and validate the model. The addition of years of retrospective data would probably slightly improve the forecast accuracy at the national level but might greatly improve precision at the regional level.

Value of Additional ILI Surveillance System

Our findings confirm previous studies that demonstrate the utility of using drug sales, and the timing of drug sales, compared to other indicators (*8,15*), as a proxy indicator of ILI activity. Several arguments support the need to consider syndromic surveillance based on drug-sales data. First, the nonspecific prodrome phase of many diseases may be self-treated before persons see a health practitioner and may therefore be more easily detected by using drug sales than laboratory surveillance or health center discharges. Second, rapidly extending the use of this method may be more feasible than creating or expanding sentinel networks of general practitioners. Drug sales are usually available in many developed countries, whereas electronic real-time surveillance of influenza or ILI is still seldom set up in most parts of the world. Third, using several sources of data with different methodologic approaches for syndromic surveillance may improve detection and prediction of trends of ILI outbreaks caused by influenza or other emerging agents. Drug-sales series represent an independent source of information, as well as reports from laboratories, general practitioners, hospitals, and death certificates, which have proved their usefulness in monitoring and assessing the impact of influenza epidemics.

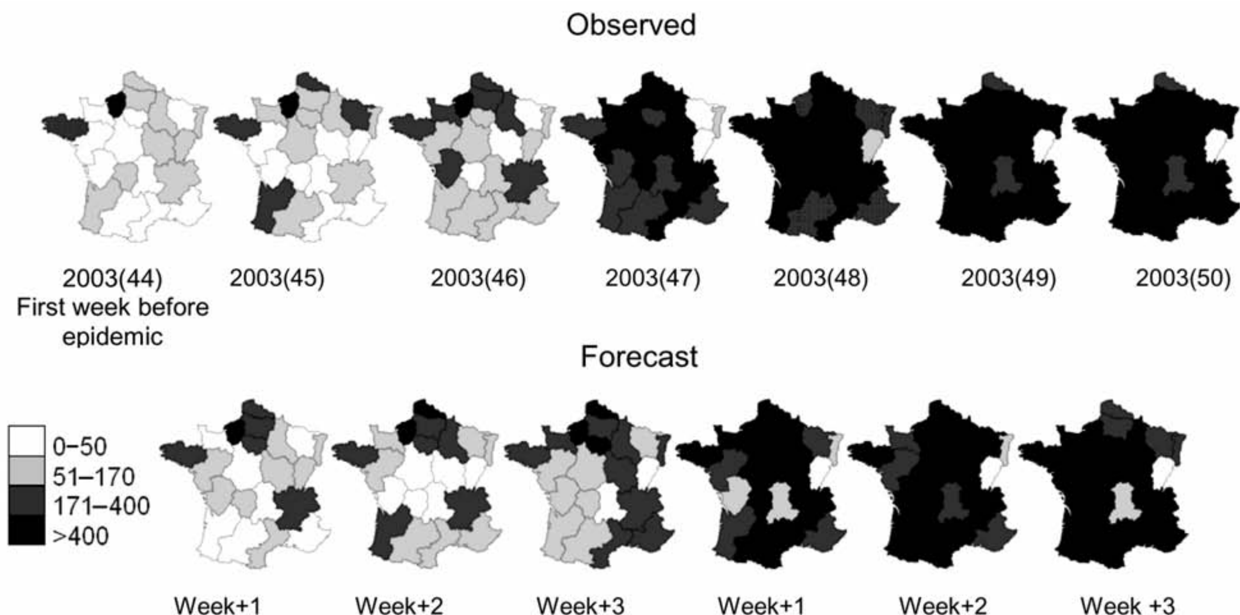

Figure 3. Evolution of regional influenzalike illness (ILI) incidence during the 2003–2004 epidemic. The observed maps (first line) were constructed by using data from the French Sentinel Network. The forecast maps (for the first 6 epidemic weeks) 1, 2, and 3 weeks ahead show the results of the regional models when medication sales are used. The forecast horizon is indicated below each map. For example, for 2003(49), ILI predicted incidence is calculated by employing the model with data until week 47 of year 2003. Thus, the time forecasting horizon is 2 weeks.

Accuracy of Drug Sales–based Surveillance System

Methods for assessing the quality of a syndromic surveillance system have been recently proposed by Buckeridge et al. (*16*). Our drug-sales time series was too short to allow a precise assessment of system's capacity to detect outbreaks appropriately. Our findings do indicate, however, that drug sales are good predictors of ILI activity recorded by the sentinel system. FSN has monitored ILI activity in France with the same method since 1984. For 21 years, during each winter, an influenza epidemic has been detected by the 2 French national influenza centers (based in Lyon and Paris) on the basis of virus isolation and simultaneously by FSN. Thus, FSN has shown a high sensitivity to detect national influenza epidemics, and we may assume that the system based on drug sales will be at least as sensitive as that of FSN. A potential advantage of the medication sales data is that their broad scope may enhance the sensitivity of detection, especially at a local level. This hypothesis has to be further assessed by evaluating a longer time period or by using simulated data for evaluation. Although using drug sales as a monitoring tool has clear benefits, detecting a nonspecific signal from our system would require further confirmation and identification of the causes of this unusual increase.

Conclusions

Our results confirm that drug-sales data could be used as an independent additional source of information to warn of ILI outbreaks early in countries where influenza is already monitored. Drugs-sales data may be the only monitoring ILI system in countries without existing surveillance systems. The proposed method has the advantage of being both practical and relatively simple to implement. Therefore, this approach could be easily extended to other infectious diseases. In many industrialized countries, similar databases of medication sales are available in real or near-real time.

This research was conducted during the postdoctoral studies of Dr Vergu and Dr Grais in the Epidemiology, Information Systems, Modeling Unit 707 at the French National Institute for Medical Research.

Dr Vergu is a biomathematician in the Applied Mathematics and Computer Science Unit at the French National Institute for Agricultural Research. She works on modeling different aspects of the epidemiology of infectious diseases.

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Personal Hygiene and Methicillin-resistant *Staphylococcus aureus* Infection

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Methicillin-resistant *Staphylococcus aureus* (MRSA) infections outside the healthcare setting are an increasing concern. We conducted a case-control study to investigate an MRSA outbreak during 2002–2003 in a Missouri prison and focused on hygiene factors. Information on sociodemographic characteristics, medical history, and hygiene practices of study participants was collected by interview and medical record review. Logistic regression was used to evaluate MRSA infection in relation to hygiene factors individually and as a composite hygiene score; potential confounding factors were controlled. Selected MRSA isolates were analyzed by pulsed-field gel electrophoresis (PFGE). MRSA infection was significantly associated with a low composite hygiene score. Transmission among prison inmates appeared to be responsible for this outbreak. PFGE analysis showed that isolates were indistinguishable and associated with community-onset MRSA infections in other US prisons. Improving hygiene practices and environmental conditions may help prevent and interrupt future MRSA outbreaks in prison settings.

In recent years, outbreaks of methicillin-resistant *Staphylococcus aureus* (MRSA) infection have been reported in different settings, including among athletic teams and military recruits, as well as in nursing homes and correctional facilities (*1–5*). A number of risk factors for MRSA infection have been identified in these studies, including antimicrobial drug use, close contact with persons colonized with MRSA, and barriers to medical care. Although some outbreak investigations have pointed to personal hygiene as a risk factor for MRSA infection (*1,4*), few studies identified specific personal hygiene practices associated with increased risk.

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In June 2003, an outbreak of MRSA infection at a women's correctional facility (prison X) was reported to the Missouri Department of Health and Senior Services. As part of the investigation and control of this outbreak, a case-control study of risk factors for MRSA infection was conducted, with a focus on personal hygiene factors. In addition, a laboratory investigation of the specimens collected from the infected persons was conducted to identify the strain of *S. aureus* implicated in the outbreak by pulsed-field gel electrophoresis (PFGE). This report summarizes the results of the investigation.

Materials and Methods

Case-Control Study

A case-control study was conducted to examine risk factors for MRSA infection in this outbreak, with a focus on personal hygiene factors. A case was defined as an inmate at prison X with culture-confirmed MRSA infection of skin or soft tissue diagnosed between January 1, 2002, and May 30, 2003. Controls were randomly chosen at the same prison with a systematic random sampling scheme from inmates who never experienced illness compatible with MRSA infection during the study period, and whose physical examination at the time of the investigation showed no evidence of MRSA infection. Case-patients and controls who reported having skin or soft tissue infection at prison admission were excluded. A trained interviewer administered a face-to-face interview with a standard questionnaire, and a physician performed a brief physical examination at the time of the interview. The interviewer collected information about sociodemographic characteristics, relevant medical history, personal hygiene factors (including hand washing, shower, laundry practices, and sharing personal items), use of gymnasium and

barbershop, and attending educational classes. Medical records for the case-patients were obtained from the prison clinic where they sought care. Additionally, information regarding the history of antimicrobial drug use, chronic medical conditions, history of hospitalization, and history of skin infection at prison arrival was obtained through the interview and medical record review. Being overweight in this study was defined as a body mass index (BMI, weight in kilograms divided by height in meters squared) ≥25 (*6*).

To evaluate an overall effect of personal hygiene practice on MRSA infection, a composite hygiene score was created on the basis of the sum of scores of 3 individual hygiene practices, including frequency of hand washing per day (1 = <6 times, 2 = 6–12 times, 3 = >12 times), frequency of a shower per week (1 = <7 times, 2 = 7–13 times, 3 = >13 times), and number of personal items shared with other inmates (1 = ≥2 items, 2 = 1 item, 3 = none). In this manner, lower composite hygiene scores indicated poorer personal hygiene practices. For this study, a composite hygiene score ≤6, which corresponds to the 25th percentile for the distribution of the composite score, was categorized as poor personal hygiene practice. Sociodemographic characteristics, medical conditions, hygiene practices, and other potential risk factors were compared between patients and controls by using the χ^2 test or Fisher's exact test (*7*). Logistic regression was used to evaluate crude and adjusted odds ratios (aORs) and their associated 95% confidence intervals (CIs) for MRSA infection in relation to individual hygiene factors separately; we controlled for patient age, race, educational level, being overweight, and skin condition before arrival at the prison. The final logistic regression model included the composite hygiene score and the covariates listed above. Model parameters were estimated by using the maximum likelihood method; their statistical significance was assessed by using the Wald statistic (*8*). All statistical analyses were performed with SAS version 9.1 (SAS, Cary, NC, USA) (*9*).

Laboratory Investigation

Genotyping on selected MRSA isolates was performed by using PFGE with *SmaI*-digested DNA. Gels were analyzed with BioNumerics software (Applied Maths, Kortrijik, Belgium) as described by McDougal et al. (*10*). Pulsed-field types were defined in a national database as having >80% similarity in a dendrogram derived from the unweighted pair group method with arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.25% and 0.5%, respectively.

Written consent was obtained from all study participants. The Institutional Review Board at the Missouri Department of Health and Senior Services reviewed and approved this study.

Results

A total of 55 culture-confirmed MRSA cases occurred at prison X during the study period (Figure 1). Of those case-patients, 30 (55%) were available for interview at the time of the investigation; 82 inmates of the same prison who had no MRSA infection were randomly selected as controls. All 30 patients participating in the study had skin or wound infections. Two controls were excluded from the study; 1 had folliculitis and the other provided insufficient data during the interview. The final dataset contained 30 cases and 80 controls.

The average length of stay in the prison, calculated from admission to the time of outbreak investigation, was significantly lower for case-patients (22.8 months) than for controls (38.9 months). The mean time from prison admission to culture confirmation of MRSA infection was 624 days (range 48–2,303), and the median was 415 days; 27 (90%) of 30 had culture confirmation ≥90 days after prison admission.

Compared with controls, patients were younger (mean age 34.5 vs. 41.5 years), more likely to be African American or American Indian, and less likely to have attended college (Table 1). No statistically significant differences between cases and controls occurred with regard to chronic medical conditions, hospitalization during the 6 months before prison admission, and intravenous drug use. Case-patients were more likely than controls to report abnormal skin conditions (i.e., infection, dermatitis, eczema; 13.3% vs. 3.8%) in their medical history, but the difference was marginally significant (p = 0.09). Patients and controls did not differ in antimicrobial drug use (topical or systemic) in the 3 months before prison admission. Patients were significantly more likely to be overweight (56.7% vs. 23.8%, p<0.01) than controls (Table 1). Patients and controls did not differ significantly in use of the gymnasium and barbershop and in attending classes.

When personal hygiene factors were examined for cases and controls (Table 2), patients were more likely than controls to share personal products (e.g., cosmetic items, lotion, bedding, toothpaste, headphones), especially nail clippers (26.7% vs. 10%, p = 0.04) and shampoo (13.3%

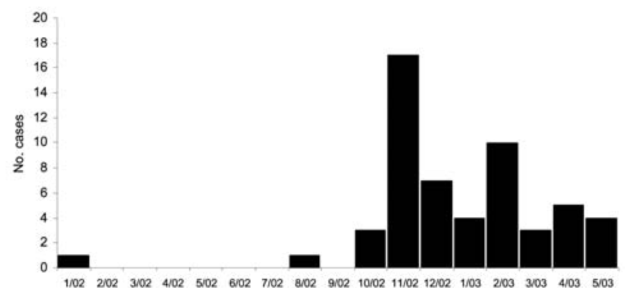

Figure 1. Onset dates of culture-confirmed cases of methicillin-resistant *Staphylococcus aureus*, prison X, Missouri, 2002–2003.

Table 1. Characteristics of patients with methicillin-resistant *Staphylococcus aureus* infection and controls, prison X, Missouri, 2002–2003*

Characteristics	Case-patients, % (n = 30)	Controls, % (n = 80)	Crude OR (95% CI)
Age, y			
20–34	60	20	6.00 (1.47–24.45)
35–49	30	60	1.00 (0.24–4.15)
≥50	10	20	1.00
Race			
Caucasian	20	51.3	1.00
Non-Caucasian	80	48.8	4.21 (1.55–11.39)
Educational level			
No college†	86.7	72.5	2.47 (0.77–7.88)
College	13.3	27.5	1.00
Overweight‡			
Yes	56.7	23.8	4.20 (1.73–10.19)
No	43.3	76.3	1.00
Antimicrobial drug use in the 3 months before imprisonment			
Yes	13.3	7.5	1.90 (0.50–7.26)
No	86.7	92.5	1.00
Abnormal skin condition before arriving			
Yes	13.3	3.8	3.95 (0.83–18.82)
No	86.7	96.3	1.00

*OR, odds ratio; CI, confidence interval. Other characteristics examined included chronic medical condition, daily medication use for treating chronic conditions, hospitalization 6 months before prison admission, intravenous drug use, use of gymnasium or barbershop, and attending educational classes. These characteristics did not differ significantly between patients and controls.
†Included no high school, graduation from high school, or general educational development.
‡Defined as a body mass index ≥25.

vs. 1.3%, p = 0.02), with other inmates. Patients were also less likely than controls to wash personal items (80.0% vs. 88.8%, p<0.01) or bed linens (26.7% vs. 52.5%, p<0.01) themselves instead of using the prison laundry. Additionally, patients tended to wash their hands and take showers less often.

When personal hygiene factors were examined individually by logistic regression while controlling for sociodemographic and other risk factors, inmates who used the prison laundry to wash their personal items (aOR 23.89, 95% CI 2.1–275.9) or bed linens (aOR 3.9, 95% CI 1.3–12.0) were more likely to have an MRSA infection than inmates who washed those items themselves. Because 8 controls were unsure about this question and were excluded from this analysis, we performed a sensitivity analysis by assigning them to the group using the prison laundry to produce the most conservative estimates. In so doing, washing bed linen in the prison laundry still showed a marginally significant association with MRSA infection (aOR 2.84, 95% CI 0.96–8.42), whereas the effect of washing personal items in the prison laundry was largely diminished (aOR 1.74, 95% CI 0.46–6.60).

The risk for MRSA infection also increased with lower frequency of hand washing per day and showers per week. Inmates who washed their hands 6–12 times (aOR 3.27, 95% CI 1.10–9.76) and <6 times (aOR 2.17, 95% CI 0.15–31.93) per day had an increased risk for infection compared with that of inmates who washed their hands >12 times per day. Inmates who took <7 showers per week (aOR 5.01, 95% CI 0.53–47.11) and those who took 7–13 showers per week (aOR 2.68, 95% CI 0.85–8.46) had an increased risk for infection compared with that of inmates who took ≥14 showers per week. Inmates who shared personal products with other inmates tended to have an increased risk for MRSA infection compared with inmates who did not share their personal products with others.

When the data were examined by using the composite hygiene score, a significantly higher proportion of case-patients than controls had lower hygiene scores (≤6) (46.7% vs. 20.0%, p<0.01). When the relationship between MRSA infection and the composite hygiene score was evaluated while simultaneously controlling for sociodemographic characteristics and other risk factors (Table 3), inmates who had poor composite hygiene scores (≤6) had a significantly higher risk for MRSA infection compared with those who had higher composite hygiene scores (aOR 3.14, 95% CI 1.1–8.93). The risk for infection also tended to increase with being younger, overweight, and nonwhite, and having a history of an abnormal skin condition. When case-patients were asked how they cared for their skin infections, 53.3% reportedly did not always cover their skin lesions, 36.7% did not have extra dressing for changing when needed, 56.7% picked their sores, and 36.7% did not change their dressings daily.

Analysis of PFGE results showed that 6 isolates had indistinguishable banding patterns and were identified as

Table 2. Distribution of hygiene factors among persons with methicillin-resistant *Staphylococcus aureus* and controls, prison X, Missouri, 2002–2003*

Characteristics	Case-patients, % (n = 30)	Controls, % (n = 80)	Adjusted OR (95% CI)
Always wash personal items themselves			
Yes	80	88.8	1.00
No	20	1.3	23.89 (2.07–275.88)
Not sure	0	10	
Always wash bed linen themselves			
Yes	26.7	52.5	1.00
No	73.3	37.5	3.88 (1.25–12.01)
Not sure	0	10	
Share any product (cosmetics, nail clipper, shaver, bedding, etc.)			
Yes	60	37.5	1.79 (0.64–4.99)
No	40	62.5	1.00
No. shared products			
≥2	33.3	17.5	2.15 (0.63–7.39)
1	26.7	20	1.49 (0.44–5.11)
0	40	62.5	1.00
Share shampoo			
Yes	13.3	1.3	3.32 (0.30–36.67)
No	86.7	98.8	1.00
Share nail clipper			
Yes	26.7	10	3.03 (0.85–10.74)
No	73.3	90	1.00
Wash hands, times per day			
<6	6.7	2.5	2.17 (0.15–31.93)
6–12	50	32.5	3.27 (1.10– 9.76)
>12	43.3	65	1.00
Showers per week			
<7	10	5	5.01 (0.53–47.11)
7–13	66.7	53.8	2.68 (0.85–8.46)
≥14	23.3	41.3	1.00

*OR, odds ratio; CI, confidence interval. Adjusted ORs were from separate logistic regression models in which the individual hygiene factor and age, race, educational level, overweight (body mass index ≥25), and abnormal skin conditions before arrival were included.

USA300.0114 (Figure 2). The banding pattern of a seventh isolate (SA15) differed by 1 band from the outbreak pattern.

Discussion

In this case-control study of an MRSA outbreak in a prison setting, poor personal hygiene practices were significantly associated with an increased risk for MRSA infection after controlling for sociodemographic and other risk factors. This outbreak was likely caused by transmission inside the prison because 90% of the case-patients had culture confirmation at least 90 days after prison admission, and subtyping by PFGE showed that 6 of the 7 isolates tested had identical PFGE patterns and 1 differed by only 1 band. These isolates belonged to pulsed-field type USA300 lineage, which is associated with community-onset MRSA infections in other correctional facilities and community outbreaks (*11*).

Based on literature review, outbreaks of MRSA infection are thought to be caused by the complex interaction of the environment contaminated by MRSA, indiscriminate use of antimicrobial drugs, and personal hygiene factors (*12,13*). In a crowded, institutionalized setting such as a prison, the interplay of such factors is more pronounced. As a result, many outbreaks have occurred in such settings (*1,14*). Hospital environmental surfaces, healthcare worker gowns, and patient-care items contaminated by patients infected or colonized with MRSA have been shown to pose significant risks for MRSA transmission (*12,15*). Boyce et al. (*16*) found that 73% of hospital rooms containing patients infected with MRSA and 69% of rooms containing patients colonized with MRSA had environmental contamination. Research also showed that the nurses' gloves became contaminated 42% of the time after they touched surfaces contaminated with the bacteria. Potential transmission of MRSA infection through contaminated surfaces and shared items was identified in a rural community by Baggett et al. (*17*). In a community-based study, Calfee et al. (*18*) demonstrated that close contact with a person colonized or infected with MRSA resulted in a 7.5-fold greater risk of becoming colonized with MRSA. Persons colonized with MRSA also have an increased risk for MRSA infection (*19,20*). Based on the results of these studies and observations in this study, one can conclude

Table 3. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) associated with risk factors for infection with methicillin-resistant *Staphylococcus aureus*, prison X, Missouri, 2002–2003*

Characteristics	Adjusted OR (95% CI)
Composite hygiene score	
≤6	3.14(1.10–8.93)
>6	1.00
Age, y	
20–34	3.57 (0.70–18.19)
35–49	0.75 (0.16–3.60)
≥50	1.00
Race	
Caucasian	1.00
Non-Caucasian	2.21 (0.70–6.96)
Educational level	
No college†	1.22 (0.30–4.92)
College	1.00
Overweight‡	
Yes	2.48 (0.86–7.14)
No	1.00
Abnormal skin condition before arriving	
Yes	2.65 (0.47–15.07)
No	1.00

*Adjusted ORs were calculated from a logistic regression model that simultaneously included all of the risk factors shown.
†Included no high school, graduation from high school, or general educational development.
‡Defined as a body mass index ≥25.

that a prison environment can be easily contaminated by MRSA. Improved personal hygiene may provide protection for inmates living and working in such contaminated environments.

In this outbreak, a complex set of factors likely contributed to the spread of infection. These factors include improper care of infected skin lesions by inmates, poor personal hygiene by inmates, and close contact in confined space.

Risk factors in an MRSA outbreak in a Georgia prison were previous antimicrobial drug use, self-draining of boils, skin lacerations, washing clothes by hand, sharing soap, and recent arrival at the prison; risk factors in an MRSA outbreak in a Texas prison included previous skin infections and recent contact with MRSA-infected persons (*1*). Nguyen et al. (*21*) found that sharing soap was associated with recurrent MRSA infections in a football team. Our finding that sharing personal hygiene items is a risk factor for MRSA infection is consistent with these observations. The use of antimicrobial drugs within 3 months before incarceration did not appear to be a significant risk factor in our investigation, and prior skin conditions, including infections, were only marginally associated with MRSA infection by univariate analysis.

Previous research indicated that patients with community-acquired MRSA infections are usually children and young adults (*13*). Our study also indicates that younger age appears to be associated with an increased risk for MRSA infection in a prison setting. The increased risk associated with younger inmates in our study was likely due to a more active lifestyle, which predisposes them to skin abrasions. These abrasions serve as the ports of entry for bacterial infection.

Our study found that being overweight was a risk factor for MRSA infection. This finding was consistent with the results of a study by Kazakova et al. (*11*), who reported a significantly higher risk for MRSA infection in football players with a higher BMI. In a study on postoperative mediastinitis caused by methicillin-susceptible *S. aureus* conducted by Duke University Medical Center, the only independent risk factor was obesity (*22*). Persons who are overweight may have different patterns of skin colonization with MRSA, which puts them at greater risk for MRSA infection.

Previous studies indicated that certain racial and ethnic minority groups may have higher rates of colonization and infection with community-acquired MRSA (*23,24*). In our study, being nonwhite (African American and American Indian) was a significant risk factor for acquiring MRSA infection before controlling for other risk factors. However, after controlling for other risk factors, this association was no longer significant.

Several limitations should be considered when interpreting the findings of this study. First, the MRSA cases were diagnosed between January 1, 2002, and May 30, 2003. However, all questionnaires were administered in May 2003. The length of time from symptom onset to the date of interview was 0.5–17 months (median 4.9). Therefore, there could be recall bias about risk factors, especially for inmates who were interviewed long after symptom onset. Second, all study participants were incarcerated adult women. Therefore, the study findings may not be applicable to other populations. Third, personal hygiene factors emerged as a leading factor for MRSA transmission in a prison setting. Whether this finding can be applied to other settings (e.g., hospitals, nursing homes, and communities) needs further investigation.

Figure 2. Dendrogram of the general relatedness (scale bar) of a sample of methicillin-resistant *Staphylococcus aureus* isolates based on pulsed-field gel electrophoresis of *Sma*I-digested DNA and comparisons of banding patterns using Dice similarity coefficients.

The findings of this study underscore the importance of the targeted education efforts to control MRSA outbreaks. Education about MRSA infection, especially the importance of proper personal hygiene, should be an integral part of efforts to eliminate and prevent MRSA infections and outbreaks. Such measures may be important in reducing the spread of MRSA in prison settings, where inherent rules and regulations complicate the implementation of certain control measures.

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Rickettsia prowazekii and Real-time Polymerase Chain Reaction

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Rickettsia prowazekii is the causative agent of epidemic typhus and a potential bioterrorism agent. Sensitive and specific rapid assays are needed to complement existing methods of detecting this organism. We developed a real-time quantitative polymerase chain reaction assay by using a species-specific probe targeting the *gltA* gene. This assay, which was rapid, specific for *R. prowazekii* only, and sensitive (cutoff detection of 1 to 5 copies per sample), detected and directly identified *R. prowazekii* in blood of 12 experimentally infected mice sampled at day 3 and 6 postinfection or in naturally or experimentally infected lice. Because our assay is highly standardized and easily adaptable, it could improve epidemic typhus surveillance in public health programs, especially for countries with underdiagnosed or unrecognized human cases.

Rickettsia prowazekii is the causative agent of epidemic or louseborne typhus, which is transmitted by the human body louse. This disease can be fatal and, without treatment with doxycycline, will cause death in up to 30% of cases (*1–3*). More than 30 million cases of epidemic typhus occurred during and immediately after World War I, resulting in an estimated 3 million deaths (*1*). Although the incidence of epidemic typhus is low today, the infection could reemerge in epidemic form in human populations, as recently reported in Burundi (*4*), Russia (*5*), and Algeria (*6*). Infections with *R. prowazekii* have been rarely reported in the United States: only 39 cases were reported from 1976 to 2001, all in persons who had no reported contact with body lice but did have contact with flying squirrels (*7,8*).

The ability to be acquired by the aerosol route, efficient arthropod transmission, and severe clinical outcome and death in untreated cases make *R. prowazekii* a category B bioterrorism agent. The former Soviet Union's Red Army developed *R. prowazekii* as a battlefield weapon, and the Japanese army successfully tested bombs containing the pathogen (*9*).

Since clinical signs of epidemic typhus are usually nonspecific, including fever, headaches, and severe myalgia, appropriate diagnostic methods are important (*10*). Despite recent developments in cell culture and molecular detection methods for the diagnosis of rickettsial diseases (*11*), serologic assays remain the simplest diagnostic tests to perform, even if serum samples are sent on filter paper (*12*). Nevertheless, serologic tests lack specificity because most also detect cross-reactive antibodies among the typhus-group rickettsioses. Moreover, a definite diagnosis of epidemic typhus is often delayed because the sensitivity of cell culture and polymerase chain reaction (PCR) methods is low (*13*), and serologic diagnosis can be obtained only by using advanced serologic methods such as Western blot analysis after cross-adsorptions. These methods are restricted to laboratories with biosafety level 3 (BSL-3) facilities and trained technicians (*14*). Recent studies have demonstrated the usefulness of PCR of body lice in ongoing surveillance of louse-associated infections, especially in outbreaks of epidemic typhus (*15*). The aim of our study was to develop a real-time quantitative PCR assay by using a species-specific probe that is rapid, sensitive, and specific for detecting *R. prowazekii* in clinical samples or in body lice in outbreaks of epidemic typhus.

Materials and Methods

The *gltA* sequences of 22 *Rickettsia* species were aligned by using the multiple-sequence alignment program ClustalW supported by the Infobiogen website (www.infobiogen.fr). Within the alignments, primers and probe were selected that were specific for *R. prowazekii*.

R. prowazekii strain Breinl (ATCC VR-142) was grown in Vero cell monolayers cultured in minimal essential medium supplemented with 4% fetal calf serum and 2 mmol/L L-glutamine as previously described (*16*). Infected cells were harvested by using sterile glass beads

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and sonicated. Cell fragments were removed by centrifugation, and the supernatant was centrifuged for 10 min at 7,500 × *g*. The resulting pellet was resuspended in 20 mL phosphate-buffered saline, pH 7.5. *R. prowazekii* inoculum was quantified by using either the plaque assay method (*17*) or comparatively by 10-fold serial dilutions of a known plasmid standard of *R. prowazekii* containing 2.0 × 10^7 copies per sample in an independent real-time PCR as previously described (*18*).

In this assay 4 *R. prowazekii* strains, 21 strains of *Rickettsia* spp., and 14 strains of bacteria from genera other than *Rickettsia* were evaluated (Table). We also included 31 lice from an outbreak of epidemic typhus in Rwanda in 2004 and 10 *R. prowazekii* laboratory-infected

Table. Strains used in real-time PCR*

Strains	Source	Standard PCR	LC PCR assay
Rickettsia prowazekii Breinl	ATCC	+	+
R. prowazekii Evir	UR	+	+
R. prowazekii BatnaRp22	UR	+	+
R. prowazekii Russian sample	UR	+	+
R. typhi Wilmington	ATCC	+	–
R. massiliae Mtul	ATCC	+	–
R. montanensis	ATCC	+	–
"*R. aeschlimanii*"	UR	+	–
R. massiliae strain Bar 29	UR	+	–
R. helvetica C6P9	ATCC	+	–
R. felis	UR	+	–
"*R. sibirica mongolitimonae*"	UR	+	–
R. rickettsii	ATCC	+	–
R. conorii moroccan	ATCC	+	–
R. sibirica sibirica 246	ATCC	+	–
R. conorii subsp. *israelensis* CDC1	G.A. Dasch	+	–
R. africae ESF–5	UR	+	–
R. japonica YM	ATCC	+	–
Thai tick typhus rickettsia	G.A. Dasch	+	–
R. slovaca	UR	+	–
R. conorii subsp. *caspia* A-167	UR	+	–
R. australis Phillips	G.A. Dasch	+	–
R. honei RB	GRIC	+	–
Rickettsia sp. AT1	UR	+	–
R. bellii 369L42-1	D.H. Walker	+	–
31 lice from Rwanda (2004)	J. Bosco	+ (10/31)†	+(17/31)†
30 lice from Rwanda (2005)	J. Gatabazi	ND	+(5/30)
R. prowazekii laboratory-infected lice	UR	+ (10/10)	+(10/10)
R. typhi laboratory-infected lice	UR	+ (10/10)	–
R. prowazekii–infected BALB/C mice	UR	+ (12/12)‡	+ (12/12)‡
B. recurrentis laboratory-infected lice	UR	– (0/10)	– (0/10)
Borrelia recurrentis	ATCC	–	–
Escherichia coli	CIP	–	–
Proteus mirabilis	CIP	–	–
Staphylococcus aureus	CIP	–	–
Streptococcus salivarius	CIP	–	–
Orientia tsutsugamushi	CIP	+	–
Streptococcus pyogenes	CIP	–	–
Mycobacterium xenopi	CIP	–	–
Chlamydia trachomatis	Human isolate	–	–
Propionibacterium acnes	UR	–	–
HGE agent	ATCC	–	–
Bartonella quintana Oklahoma	ATCC	–	–
Tropheryma whipplei Twist	UR	–	–
M. tuberculosis	CIP	–	–

*PCR, polymerase chain reaction; LC, LightCycler; ATCC, American Type Culture Collection, Rockville, MD, USA; GRIC, Gamaleya Research Institute Collection; G.A. Dasch, Naval Medical Research Institute, Bethesda, MD, USA; UR, Unité des Rickettsies, CNRS UPRES A, Marseille, France; D.H. Walker, University of Texas, Galveston; CIP, Collection Institute Pasteur, Paris, France; HGE, human granulocytic ehrlichiosis.
†Number of positive lice/total number of tested lice.
‡PCR for each mouse was positive in blood at days 3 and 6 postinfection (for cycle thresholds see results in text).

lice (*19*), 10 *R. typhi* laboratory-infected lice (*20*), and 10 *Borrelia recurrentis* laboratory-infected lice (*21*). Finally, we also included 30 lice received in June 2005 from Rwanda, which were tested only with the quantitative PCR (qPCR) assay (Table). Negative controls included 10 pathogen-free lice, distilled sterile water, and PCR mixture. All experiments were repeated 4 times. For mice samples, DNA samples extracted from blood of uninfected mice were used as negative controls.

We have also tested *R. prowazekii*–infected mice by using a currently available experimental model similar to the previous model described for *R. typhi* (*22*). We used 7-week-old female BALB/C mice (Charles River Laboratories, Arbresle, France) that were maintained in cages with sterile food and water. All experiments were performed in a BSL-3 laboratory. Twelve mice were injected with 1.8×10^5 PFU/mL *R. prowazekii* strain Breinl (ATCC VR-142), and 6 mice were injected with uninfected cells. The solution containing bacteria was injected into the retroorbital venous plexus over a period of 30 s. We collected 200 µL of blood from each mouse at day 3 postinfection (PI) and at day 6 PI and stored it in EDTA at –20°C for PCR.

Total genomic DNA from bacterial strains was extracted with the Qiagen QIAamp Blood Kit (Qiagen, Hilden, Germany), and lice DNA and blood and biopsy samples from infected mice were extracted by using the Qiagen QIAamp Tissue Protocol (Qiagen). PCR was performed by using a LightCycler instrument (Roche Biochemicals, Mannheim, Germany).The PCR mixture included a final volume of 20 µL with 10 µL of the Probe Master kit (Qiagen), 0.5 µL (10 pmol/µL) of each primer, 2 µL (2 µmol/µL) probe, 5 µL distilled water, and 2 µL extracted DNA. The amplification conditions were as follows: an initial denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C, annealing and elongation at 60°C for 120 s, with fluorescence acquisition in single mode. Each sample was also tested with a standard PCR that was performed on a PCR instrument (Eppendorf, Mastercycler, Hamburg, Germany) using primers of the *gltA* gene (*23*).

Results

The *R. prowazekii* inoculum used in this study was of 1.8×10^5 PFU/mL using the plaque assay quantification method (*17*) and contained 1.16×10^6 copies per sample when quantified with our plasmid standard (*18*). The selected primers and probe of the *gltA* gene specific only for *R. prowazekii* were as follows: RproF (5′-TCGGTAAAGATGTAATCGATATAAG-3′), RproR (5′-CATATCCTCGATACCATAATATGC-3′) and Rp.probe (FAM-ACTTTACTTATGATCCGGGTTTTATG-TAMRA), leading to a PCR product size of 154 bp. When qPCR assay was used, only *R. prowazekii* strains were positive, whereas the standard PCR assay detected all rickettsial species (Table). The standard PCR assay was positive for all 20 laboratory-infected lice (*R. typhi* or *R. prowazekii*), while qPCR assay was positive only for the 10 *R. prowazekii* laboratory-infected lice. Finally, blood samples obtained from our experimental model of *R. prowazekii*–infected mice at days 3 and 6 PI were also positive by using the protocol described above. The mean number of cycle thresholds (Ct value) for mice sampled at day 3 PI was 32.47 ± 2.11; at day 6 PI, the Ct value was of 35.52 ± 2.01 ($p = 0.001$). All uninfected lice, *B. recurrentis*–infected lice, and mice samples were negative with both assays.

The sensitivity of qPCR and the standard PCR was determined by using 10-fold serial dilutions of our known *R. prowazekii* inoculum (1.16×10^6 DNA copies per sample). The sensitivity of the qPCR was increased 10-fold over that of the standard PCR. Compared to our plasmid standard, the cutoff detection of the qPCR was 1–5 copies per sample, whereas the cutoff detection was >10 copies for the standard PCR.

Among the 31 lice from Rwanda sampled in 2004, 17 were positive by real-time PCR, whereas only 10 of these 17 lice were positive by standard PCR. The latter 10 samples had a mean number of 1,300 DNA copies (Ct value 26.82–35.22). The 7 samples positive only by real-time PCR had a mean number of 8.5 DNA copies (Ct value 33.72–38.73). The real-time PCR therefore appears to be more sensitive. However, this difference was not significant ($p = 0.07$) perhaps because of the small number of tested lice. Finally, 5 of the 30 lice received from Rwanda in June 2005 were positive when the qPCR was used (Table).

Discussion

We developed a real-time quantitative PCR for specific detection of *R. prowazekii*. The selected primers and probe were 100% complementary to *R. prowazekii* only and to no other rickettsial strains. We confirmed the specificity of these primers and probe on rickettsial isolates and other common bacteria and repeated the experiments 4 times without discrepancies. Real-time quantitative PCR for rickettsiae was first developed to test antimicrobial drug susceptibility (*24*) and then was used to detect *R. rickettsii* and closely related spotted fever group rickettsiae (*17*) or *R. prowazekii* strains (*25*).

Our assay has a greater sensitivity than the standard assay, with a cut-off detection of only 1 to 5 DNA copies per sample, as measured comparatively to plasmid DNA quantification. The sensitivity found with our standard PCR has been previously estimated at 1–10 DNA copies of the gene (*23*). The first use of standard PCR for detecting

R. prowazekii using primers derived from the 17-kDa antigen sequence had a cut-off detection of as few as 30 rickettsiae (*26*). Cutoff detection of rickettsiae with real-time quantitative PCR ranges from 5 copies (*17*) to 10 copies (*25*). Using our LightCycler assay, we detected an extra 7 samples in lice from Rwanda as compared to standard PCR. Only 1 report exists of real-time detection of *R. prowazekii* using molecular beacon probes targeting the *ompB* gene (*25*). In this report, only 2 *R. prowazekii* strains were tested (*25*). Moreover, we showed that *R. prowazekii* can be amplified from blood of experimentally infected mice. This experimental model of *R. prowazekii* infection and the ability to quantify the bacteria with the real-time PCR could be used to better study the pathogenesis of the organism. We found in this mouse model that the number of bacteria in blood was lower at day 6 PI than that at day 3 PI, which suggests that mice can eradicate infection at this dose.

The assay we describe can be performed wherever a real-time quantitative PCR machine is available. The reagents and the machine are standardized; this method gives rapid results (sequencing is not necessary) and decreases the likelihood of error. This assay was applied successfully in lice received from Rwanda in June 2005. Indeed, using our assay we were able to alert the World Health Organization of the presence of *R. prowazekii*–positive lice within 1 working day.

Because body lice and their associated diseases are generally encountered in areas where medical and biologic assistance is limited, local assessment of their roles as sources of infection is difficult. Lice are easy to collect and to transport to reference laboratories, where suitable molecular biologic approaches can be used (*23*). Although sucking lice die within 24 h of their final blood meal, the infecting bacterial DNA will remain intact for extraction for several weeks if the samples are kept dry (*15*). Upon arrival in the laboratory, the lice can be processed very quickly, and a diagnosis can be established rapidly (DNA extraction and LightCycler PCR take ≈5 h). Several weeks are necessary to obtain bacterial culture and serologic results, and those procedures do not always highlight the presence of bacteria. The usefulness of bacterial DNA detection in lice by PCR has been demonstrated by recent investigations. In central Africa, large outbreaks of lice infections occurred during civil wars in Burundi, Rwanda, and Zaire and preceded the outbreak of epidemic typhus by 2 years (*4*). Finally, our data obtained in experimentally infected mice suggest that real-time PCR could also be useful for detecting *R. prowazekii* directly from blood specimens. Because our assay is highly standardized and easily adaptable anywhere and anytime, it could improve epidemic typhus surveillance in public health programs, especially for countries with underdiagnosed or unrecognized human cases (*4*).

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Serosurvey on Household Contacts of Marburg Hemorrhagic Fever Patients

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The first major outbreak of Marburg hemorrhagic fever (MHF) outside a laboratory environment occurred in the subdistrict of Watsa, Democratic Republic of Congo, from October 1998 to August 2000. We performed a serosurvey of household contacts of MHF patients to identify undetected cases, ascertain the frequency of asymptomatic Marburg infection, and estimate secondary attack risk and postintervention reproduction number. Contacts were interviewed about their exposure and symptoms consistent with MHF. Blood samples were tested for anti–Marburg immunoglobulin G (IgG). One hundred twenty-one (51%) of 237 identified contacts participated; 72 (60%) were not known to the health authorities. Two participating contacts were seropositive and reported becoming ill after the contact; no serologic evidence for asymptomatic or mild Marburg infection was found. The secondary attack risk was 21%; the postintervention reproduction number was 0.9, consistent with an outbreak sustained by repeated primary transmission, rather than large-scale secondary transmission.

Marburg hemorrhagic fever (MHF) is a rare disease caused by the Marburg filovirus; it occurs in central, east, and southern Africa. MHF is characterized by sudden onset of fever, headache, myalgia, arthralgia, and frequently progresses to diarrhea and vomiting, hemorrhagic diathesis (petechiae, hematemesis, melena), and death (*1*). Case fatality reached 88% in a community outbreak in Uige, Angola (*2*). No vaccine or antiviral therapy is available; supportive treatment consists primarily of correcting fluid and electrolyte imbalances. The putative diagnosis is established on clinical and epidemiologic grounds and confirmed by polymerase chain reaction (PCR), antigen-capture enzyme-linked immunosorbent assay (ELISA), immunoglobulin M (IgM) ELISA, or virus isolation.

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The reservoir animal species capable of surviving Marburg infection and sustaining the virus's lifecycle has not been discovered (*3*); thus, transmission patterns from the reservoir to humans are not known. Transmission between humans occurs through direct contact with symptomatic MHF patients or with their body fluids or remains (*4*). The risk for transmission of Marburg virus is assumed to increase with the intensity of physical contact and the amount of body fluids shed, as shown for Ebola virus (*5*).

The first major community outbreak of MHF described (>150 putative cases, case fatality 83%) was in the mining village of Durba and the neighboring town, Watsa, in the northeast of the Democratic Republic of Congo (DRC), in 1999. The outbreak probably started in October 1998, had several peaks alternating with latent periods, and ended in August 2000, when the last confirmed MHF cases occurred (*6*). Primary cases were predominantly in *orpailleurs* (unofficial gold miners), while secondary cases were predominantly in household contacts and healthcare workers. Response activities similar to those for Ebola outbreaks were started in May 1999 with temporary assistance from expert teams. These measures included active and passive surveillance, follow-up of contacts, isolation of cases, barrier nursing, and safe burials (*7*).

The surveillance system likely did not identify all MHF cases because surveillance officers did not make sufficient efforts to approach families of primary case-patients, patients with mild cases were not referred to an experienced clinician for assessment (*8*), or contacts concealed symptoms compatible with MHF to avoid isolation. We carried out a serosurvey of household contacts to ascertain unidentified MHF cases and to estimate the secondary attack risk and postintervention reproduction number.

Methods

Study Area and Population

The epicenter of the 1998–2000 MHF outbreak, Durba, is 14 km from Watsa town, the administrative center of the subdistrict of Watsa. Watsa Subdistrict is located near the border with Uganda and Sudan. Watsa's health system was seriously compromised during the outbreak by economic decline and the ongoing war in eastern DRC.

Our survey was of lay persons, referred to as household contacts, whose contact with an MHF patient occurred during lay activities, such as nursing a patient (supporting, feeding, washing, and the like, whether at home or in health facilities), transporting a patient or body, or preparing a body for burial. Healthcare workers whose contact occurred during their professional duties were not eligible.

Cases of MHF were either laboratory confirmed (positive by PCR, antigen-capture ELISA, virus isolation, or a combination of IgG ELISA, IgG indirect immunofluorescence assay [IFA], and clinical and epidemiologic evidence [48 cases]) or epidemiologically linked (persons for whom laboratory confirmation was not attempted who had acute fever, hemorrhage, and contact with a laboratory-confirmed patient [25 cases]). Forty-five cases were known from surveillance during the outbreak; we identified 28 retrospectively. Contacts of suspected case-patients whose conditions were not laboratory confirmed or epidemiologically linked as defined above were not eligible because their diagnosis lacked certainty.

We attempted to visit the households of all 73 MHF patients and to prepare a list of persons who had direct contact with the patient or his or her body fluids or remains. If contacts were temporarily absent, we undertook at least 2 repeat visits. If they had moved away, we tried to locate them at their new address, unless distance or lack of security (e.g., rebel activity, bandits) hindered us, in which case we interviewed former neighbors about the contacts' disease episodes in the 4 weeks after the patient's illness. We asked all contacts we met to give verbal informed consent; if they agreed, we interviewed them and took blood samples. This study was approved by the ethics committee of the Antwerp Institute for Tropical Medicine and the representative of the Ministry of Health in Watsa.

Interviews

After establishing the identity of the contact and the relationship to the patient, we asked an open-ended question about the role the contact played during the patient's illness. We also asked closed-ended questions on whether the contact had touched, carried, or embraced the patient (and whether the patient at that point had diarrhea, vomiting, or bleeding) and whether the contact had touched the patient's clothes or linen (and whether these were soiled with stool, vomitus, or blood). Since patients who died often had had diarrhea, vomiting, and bleeding in the final stages of disease, we also asked whether the contact had touched, carried, embraced, or washed the person after death. While field testing the questionnaire, we found that protective gear such as gloves was unavailable to lay persons; thus, all contacts were assumed to be unprotected. We asked about symptoms the contact had experienced during the 4 weeks after exposure; these symptoms (Table 1) correspond to the ones used during the epidemic to define a clinically suspected case.

Blood Sampling and Testing

After the interview, 5–10 mL venous blood was taken from contacts. After 12 to 24 hours, serum was separated from the blood clot, refrigerated at ≈4°C, and transported to the Uganda Virus Research Institute (within 1 to 2 weeks). There it was frozen at –70°C and shipped on dry ice to the National Institute for Communicable Diseases, Johannesburg. Serum was examined by ELISA and IFA and considered positive if anti-Marburg IgG was found in both tests.

Data Analysis

Data were entered with EpiInfo version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA, USA), and analyzed with Stata version 8.2 software (StataCorp LP, College Station, TX, USA). Depending on symptoms associated with increased virus shedding and on the intensity of the contact, level of exposure was categorized. Low-level contact included any direct contact with a living case-patient without diarrhea, vomiting, or bleeding; or touching clothes or sheets not soiled with stool, vomitus, or blood. Medium-level contact was defined as touching a living case-patient with diarrhea, vomiting, or bleeding; touching clothes or sheets soiled with stool, vomitus, or blood; or touching remains. High-level contact included carrying or embracing a living patient who had diarrhea, vomiting, or bleeding; or carrying, embracing, or cleaning remains.

We established transmission chains and generations for all patients, taking into account work as a gold digger, exposure to other patients, incubation period, and date of onset (M. Borchert, unpub. data). When a patient had been working as a gold digger and had been exposed to another patient, we gave priority to the confirmed human-to-human exposure over the possible primary exposure and classified these cases as nonprimary ones.

We calculated the secondary attack risk as the proportion of household contacts of primary case-patients who then became secondary case-patients themselves, including only primary case-patients whose contact list could be established fully and who did not share contacts with another case-patient; we used the analogous approach to

Table 1. Symptoms in 121 household and community contacts within 4 weeks after exposure to a Marburg hemorrhagic fever patient, Watsa Subdistrict, Democratic Republic of Congo, 2002

Symptoms	No. seronegative (%), n = 119	No. seropositive, n = 2	Total (%), N = 121
Fever	37 (31.1)	1	38 (31.4)
General symptoms			
Headache	55 (46.2)	1	56 (46.3)
Fatigue	45 (37.8)	2	47 (38.8)
Loss of appetite	39 (32.8)	1	40 (33.6)
Joint pain	33 (27.7)	1	34 (28.1)
Muscle pain	26 (21.9)	1	27 (22.3)
Back pain	24 (20.2)	1	25 (20.7)
Abdominal pain	23 (19.3)	2	25 (20.7)
Chest pain	14 (11.8)	2	16 (13.2)
Nausea, vomiting	10 (8.4)	2	12 (9.9)
Diarrhea	11 (9.2)	1	12 (9.9)
Dyspnea	8 (6.7)	2	10 (8.3)
Sore throat	8 (6.7)	1	9 (7.4)
Hiccough	3 (2.5)	2	5 (4.1)
Any general symptom	68 (57.1)	2	70 (57.9)
Hemorrhage			
Nose bleed	2 (1.7)	0	2 (1.7)
Bloody/black stool	1 (0.8)	1	2 (1.7)
Coughing blood	1 (0.8)	1	2 (1.7)
Bloody vomit	0	1	1 (0.8)
Vaginal bleeding	1 (0.8)	0	1 (0.8)
Any hemorrhage	3 (2.5)	1	4 (3.3)
Combinations			
Fever + ≥3 general symptoms	32 (26.9)	1	33 (27.3)
Fever + hemorrhage	2 (1.7)	1	3 (2.5)
Clinically suspect case*	32 (26.9)	1	33 (27.3)
Total	119 (100.0)	2	121 (100.0)

*Fever + ≥3 general symptoms or fever + hemorrhage.

estimate the tertiary attack risk. We computed the reproduction number (R_p) as the product of the secondary attack risk and the average number of contacts per primary case-patient. As most cases in our survey had occurred after control measures were implemented, we consider this number to be the postintervention R_p, not the basic reproduction number R_0.

Results

Completeness of Data

Household contacts of 73 MHF patients were eligible to participate in the survey. We completed contact lists for 48 patients (66%). For 7 patients, Watsa health authorities had listed some contacts during the epidemic. Because we could not meet these patients, their contacts, or others who could verify the list's completeness we likely missed some contacts. For 18 patients, no contacts had been listed by the health authorities. Since we did not speak to anyone who had witnessed the case during the epidemic, contacts have also probably been missed for these cases.

Existing surveillance records listed 141 contacts. For the 48 cases we could investigate fully, 96 additional contacts were found. Seventy-one of these were contacts of patients identified by surveillance during the outbreak. The total number of identified contacts therefore was 237 (141 + 96), relating to 55 (48 + 7) of 73 cases.

A patient whose case was fully investigated had, on average, 4.46 contacts; on this basis, one would expect 326 contacts for all 73 patients. The 237 identified contacts correspond to 73% of this expected number. A total of 143 contacts could be traced, and 124 consented to being interviewed and giving a blood sample, representing 52% of the 237 identified contacts and 38% of the 326 expected contacts. Three persons listed by surveillance denied any physical contact with the patient and were excluded from analysis. Therefore, results refer to 121 study participants.

Characteristics of Contacts

The median interval between the onset of the patient's disease and the contact's interview and blood sample collection was 24 months (range 11–48). Half of the contacts were female, and three fourths were 15–49 years of age. Most contacts were family members (88%), while colleagues accounted for 11% (Table 2).

Half of the contacts held or carried a patient, a third fed or washed a patient, and a tenth reported sharing a bed with a patient (Table 3). Exposure to a living patient was almost

Table 2. Characteristics of 121 household or community contacts of Marburg hemorrhagic fever patients, Watsa Subdistrict, Democratic Republic of Congo, 2002

Characteristics	No. male (%), n = 63	No. female (%), n = 58	Total (%), N = 121
Age (y)			
≤4	2 (3.5)	0	2 (1.7)
5–14	3 (5.2)	5 (7.9)	8 (6.6)
15–29	24 (41.4)	35 (55.6)	59 (48.8)
30–44	20 (34.5)	11 (17.5)	31 (25.6)
≥45	9 (15.5)	12 (19.1)	21 (17.4)
Residence			
Durba	38 (65.5)	47 (74.6)	85 (70.3)
Watsa town	9 (15.5)	7 (11.1)	16 (13.2)
Other village in Watsa Health Zone	8 (13.8)	5 (7.9)	13 (10.7)
Outside Watsa Health Zone	3 (5.2)	4 (6.4)	7 (5.8)
Profession*			
Housewife	0	29 (46.0)	29 (24.0)
Unofficial gold miner	23 (39.7)	0	23 (19.1)
Pupil/student	6 (10.3)	9 (14.3)	15 (12.4)
Farmer	7 (12.1)	7 (11.1)	14 (11.6)
Trader	2 (3.5)	11 (17.5)	13 (10.7)
Health worker	2 (3.5)	2 (3.2)	4 (3.3)
Other or none	16 (27.6)	2 (3.2)	18 (14.9)
Relationship			
Spouse	3 (5.2)	12 (19.5)	15 (12.4)
Same generation as case (brother, sister, brother- or sister-in-law, cousin)	24 (41.4)	18 (28.6)	42 (34.7)
Subsequent generation (son/daughter, nephew or niece)	13 (22.4)	13 (20.6)	26 (21.5)
Preceding generation (father or mother, uncle or aunt)	7 (12.1)	16 (25.4)	23 (19.0)
Colleague	10 (17.2)	3 (4.8)	13 (10.7)
Other	1 (1.7)	1 (1.6)	2 (3.3)
Total	63 (100)	58 (100)	121 (100)

*N = 116 because of missing data.

universal; three quarters of contacts had exposure to body fluids and excreta. Forty-three percent of contacts had exposure to remains. The exposure level was low in 13%, medium in 19%, and high in 68% of contacts and did not differ between the sexes.

For 43 of the 50 contacts known to surveillance, we compared exposure reported in our survey with exposure documented by surveillance officers during the outbreak. For 88% of contacts, surveillance and study information agreed.

Two study participants were positive for anti–Marburg IgG: a 21-year-old brother and a 27-year-old male neighbor of MHF patients. Both contacts were highly exposed to their respective primary case-patients. These contacts were also, as unofficial gold miners, at risk for primary transmission themselves (*6*). The 21-year-old reported 6 general symptoms within 4 weeks after exposure, including fatigue, abdominal pain, nausea/vomiting, hiccoughs, chest pain, and difficulty breathing, but he did not fulfill the definition of a suspected case because he did not exhibit fever or bleeding. The 27-year-old reported a hemorrhagic fever syndrome, including vomiting and coughing blood and bloody or black stool. Neither contact sought medical care. We consider them to be additional confirmed patients and classified them as secondary cases because of the combination of high exposure and postexposure symptoms compatible with MHF. The 119 seronegative contacts were considered nonpatients. Thus, the overall seroprevalence in our study population is 1.65% (95% confidence interval [CI] 0.2%–5.8%), the same as in the general population (1.64%) (*6*).

Although almost all contacts were seronegative, one third reported fever within 4 weeks of contact with a patient (Table 1), more than one half reported a general symptom (headache, fatigue, and loss of appetite most frequently), and 3.3% reported hemorrhage. Thirty-three (27%) contacts would have qualified as clinically suspected case-patients during the epidemic and should have been taken to an isolation ward for assessment by an experienced healthcare worker. This did not happen, and 23 of these persons were not even known by authorities to be contacts.

On the basis of surveillance records and interviews with family members, neighbors, or colleagues of the 113 eligible contacts we could not interview or obtain blood samples from, we identified 1 epidemiologically linked patient, 1 suspected MHF case-patient, and 13 noncases; for the 98 remaining contacts, information was insufficient

Table 3. Type and level of exposure of 121 household and community contacts of Marburg hemorrhagic fever patients, Watsa Subdistrict, Democratic Republic of Congo, 2002

Exposure	No. male (%), n = 63	No. female (%), n = 58	Total (%), N = 121
Role played for the living patient			
Held or carried	33 (56.9)	31 (49.2)	64 (52.9)
Fed	14 (24.1)	24 (38.1)	38 (31.4)
Washed	14 (24.1)	24 (38.1)	38 (31.4)
Washed patient's clothes	4 (6.9)	19 (30.2)	23 (19.0)
Made patient drink	6 (10.3)	16 (25.4)	22 (18.2)
Shared bed	4 (6.9)	8 (12.7)	12 (9.9)
Give medical care	6 (10.3)	5 (7.9)	11 (9.1)
Contact with living patient			
Touched with hands	54 (93.1)	58 (92.1)	112 (92.6)
Touched a patient with diarrhea, vomiting, bleeding	40 (69.0)	44 (69.8)	84 (69.4)
Carried, embraced, or shared bed	42 (72.4)	44 (69.8)	86 (71.1)
Carried, embraced or shared bed when patient had diarrhea, vomiting, bleeding	31 (53.5)	37 (58.7)	68 (56.2)
Touched object like clothes or sheets with hand	33 (56.9)	43 (68.3)	76 (62.8)
Touched objects when soiled with stool, vomit, blood	24 (41.4)	36 (57.1)	60 (49.6)
Any physical contact with living patient or object	54 (93.1)	59 (93.7)	113 (93.4)
Any physical contact with patient or object, with putative exposure to stool, vomit, blood	41 (70.7)	49 (77.8)	90 (74.4)
Contact with remains of patient			
Touched with hands	27 (46.6)	25 (39.7)	52 (42.9)
Carried or embraced	18 (31.0)	17 (27.0)	35 (38.9)
Cleaned	9 (15.5)	7 (11.1)	16 (13.2)
Any physical contact	27 (46.6)	25 (39.7)	52 (43.0)
Level of exposure			
Low level of physical contact*	9 (15.5)	7 (11.1)	16 (13.2)
Medium level of physical contact†	10 (17.2)	13 (20.6)	23 (19.0)
High level of physical contact‡	39 (67.2)	43 (68.3)	82 (67.8)
Total	58 (100.0)	63 (100.0)	121 (100.0)

*Low, any direct contact with living patient who did not have diarrhea, vomiting, or bleeding; touching clothes or sheets not soiled with stool, vomit, or blood.
†Medium, touching living patient who had diarrhea, vomiting, or bleeding; touching clothes or sheets soiled with stool, vomit, or blood; touching remains.
‡High, carrying or embracing living patient who had diarrhea, vomiting, or bleeding; carrying, embracing, or cleaning remains.

to classify them. The total of MHF cases thus increased to 76 (50 laboratory-confirmed, 26 epidemiologically linked).

Secondary Attack Risk and Postintervention R_p

Thirty-one of 76 cases were identified as primary, 21 as secondary, 15 as tertiary, and 5 as quaternary. Four cases could not be classified. Eleven patients with secondary cases acquired their infection as a household contact and had only 1 patient with a fully investigated primary case as a possible source. These constituted the numerator for the secondary attack risk and contributed to the denominator. Forty-two healthy contacts with only 1 patient with a fully investigated primary case as possible source also contributed to the denominator. The secondary attack risk was thus estimated as 21% (11/[11 + 42], 95% CI 11–34) for household contacts. Restricting the calculation to confirmed primary cases did not significantly change the secondary attack risk estimate. The average number of household contacts per fully investigated primary case was 4.46, so that R_p for household contacts was estimated as 0.93. The tertiary attack risk (6/32 = 19%, CI 7–36) did not differ from the secondary one.

Discussion

Most of the 121 household and community contacts of MHF patients reported substantial unprotected exposure to Marburg virus through physical contact with patients, their body fluids, or remains. In addition to the secondary cases identified through surveillance, we found serologic evidence for Marburg infection in 2 persons and epidemiologic evidence in 1 person. For all 3 persons, substantial clinical disease after the exposure was reported. As most patients identified during the Watsa outbreak showed signs of disease (D.G. Bausch et al., unpub. data), we conclude that mild or asymptomatic Marburg infection, albeit possible (*8*), was a rare event.

One fourth of the seronegative contacts reported symptoms within 4 weeks of exposure, which fulfilled the definition for a suspected case. This figure illustrates the

difficulty in deciding whether to isolate patients on the basis of clinical and epidemiologic data alone. The risk for cross-contamination on the isolation ward, if persons are incorrectly hospitalized, and the risk for continued community transmission, if true cases are not isolated, show the necessity of having a laboratory diagnosis available within 1 or 2 days.

Our secondary attack risk estimate of 21% is within the range reported for Ebola outbreaks for comparable types of contacts: Ebola-Zaire, Kikwit, 1995, household contacts 16% (*5*); Yambuku, 1976, close relatives, 20% (*9*); Ebola-Sudan, Nzara, 1979, family members with physical contact including nursing 31% (*10*) Our estimate is much higher than the 2.5% reported for Ebola-Sudan, Uganda, 2000 (*11*); however, the Ugandan estimate may have included persons who merely stayed in the same house as a patient without reporting physical contact. The secondary and tertiary attack risks in our study were found to be virtually identical, 21% and 19%, respectively; thus no evidence suggested that Marburg virus loses infectivity by repeated passages through humans.

We found the postintervention reproduction number R_p to be <1; after the implementation of control measures, secondary transmission was not sustainable in the community. This finding is consistent with our observations during the outbreak, whose prolonged duration of almost 16 months after control measures were initiated in May 1999 was due to repeated primary transmission into the human population and not to sustained secondary transmission. The outbreak ended when the dominant location of primary transmission, the Gorumbwa gold mine, ceased to be accessible (D.G. Bausch et al., unpub. data). Our data do not allow computing the preintervention basic reproduction number R_0, so we cannot be certain how much of a difference the control measures made, but we think they had some effect.

The proportion of contacts (71/212) and the number of clinically suspected cases (33) missed by surveillance were high. Two of 3 retrospectively identified MHF patients were contacts of patients known to the health authorities. These contacts reported symptoms that qualified them as having suspected cases, but they were missed nevertheless. Given the importance of early recognition and isolation of MHF patients for outbreak control, this finding raises the question of how the Watsa health authorities could have been better supported in their surveillance activities. After Watsa's chief medical officer died from MHF in May 1999 (*12*), the post remained vacant for many months. We suggest that continuous support to the health zone by training and deploying a Congolese epidemiologist might have been more cost-effective than the intermittent support provided by experts from May 1999 to October 2000. This strategy would also have strengthened DRC's capacity to deal with future viral hemorrhagic fever outbreaks.

The survey's setting was characterized by high mobility because of the war and the flooding of the Gorumbwa gold mine, and we could not investigate all cases fully because of lack of available sources. For these reasons, we located at best half of all contacts and probably fewer. We could not make firm conclusions about those whom we could not interview or obtain blood from: a few may have contracted or even died from MHF. However, contacts for whom no information was available were no more likely to contract MHF than those we could study. In settings where families are isolated from their surroundings, a filovirus may wipe out a household, leaving no witness to report the event. In Durba and Watsa, where households are physically and socially close, such a tragedy is unlikely to have happened without anyone noticing, remembering, and reporting; we therefore believe that a substantial survival bias is unlikely.

The accuracy of reported exposure and symptoms may have had recall bias, given the average interval of 2 years between the patient's disease and the survey. When our data were compared with exposure information recorded by surveillance officers during the outbreak, agreement was satisfactory, however. Exposure patterns reflected traditional female and male roles in caring for diseased relatives. Since no material gains were offered to newly identified patients, we did not provide incentives to over-report exposure or symptoms. Giving a blood sample is unpopular in the study setting; to avoid underreporting exposure and symptoms, study participants were informed before the interview that a blood sample would be requested, regardless of their answers to interview questions. Those who did not wish to be interviewed or provide a blood sample refused overtly. In summary, we believe the interview data are valid.

If anti–Marburg IgG antibodies were transitory after infection with Marburg virus, they might have fallen below detectable levels in the interval between exposure and blood collection. However, samples taken from 17 MHF survivors after 22 to 102 months of follow-up that were stored, transported, and analyzed in the same way as the samples of this survey showed that none became seronegative. These persons from the 1994 or 1998–2000 MHF outbreaks became seropositive during or shortly after disease and included 2 with mild Marburg disease (M. Borchert, unpub. data). We conclude that Marburg antibodies persisted sufficiently to be detected in our serosurvey.

If some of our epidemiologically linked case-patients did not have MHF, this result could have diluted the secondary attack risk. However, restricting the analysis to confirmed cases did not increase, but rather reduced, the

secondary attack risk, albeit not significantly. We report the secondary attack risks on the basis of confirmed and epidemiologically linked cases, which is equally valid and more stable because of the larger number of observations.

Calculation of secondary attack risk and R_p depends on determining the transmission generations correctly. We are confident that our data are of sufficient quality to allow this, but an inherent uncertainty exists regarding patients who worked as unofficial gold miners and reported substantial exposure to another patient. We think these persons could be classified as having secondary cases, given the confirmed secondary, but uncertain primary, exposure. In the unlikely event that these were all primary cases, the secondary attack risk would be reduced to 16%, which would not change our conclusions substantially.

Conclusion

We found that asymptomatic or very mild Marburg infection was a rare event in the Watsa outbreak. The postintervention reproduction number R_p was <1, which suggests that the MHF outbreak in Watsa and Durba was sustained through repeated introduction of the virus into the human population and not through secondary spread. We showed that the identification and follow-up of contacts during the outbreak were incomplete and raised the question of how support for surveillance efforts in a health zone such as Watsa could be improved.

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RESEARCH

Canine Visceral Leishmaniasis, United States and Canada, 2000–2003

Zandra H. Duprey,* Francis J. Steurer,* Jane A. Rooney,* Louis V. Kirchhoff,† Joan E. Jackson,‡ Edgar D. Rowton,‡ and Peter M. Schantz*

Visceral leishmaniasis, caused by protozoa of the genus *Leishmania donovani* complex, is a vectorborne zoonotic infection that infects humans, dogs, and other mammals. In 2000, this infection was implicated as causing high rates of illness and death among foxhounds in a kennel in New York. A serosurvey of >12,000 foxhounds and other canids and 185 persons in 35 states and 4 Canadian provinces was performed to determine geographic extent, prevalence, host range, and modes of transmission within foxhounds, other dogs, and wild canids and to assess possible infections in humans. Foxhounds infected with *Leishmania* spp. were found in 18 states and 2 Canadian provinces. No evidence of infection was found in humans. The infection in North America appears to be widespread in foxhounds and limited to dog-to-dog mechanisms of transmission; however, if the organism becomes adapted for vector transmission by indigenous phlebotomines, the probability of human exposure will be greatly increased.

Visceral leishmaniasis, caused by geographic variants of the *Leishmania donovani* complex (*L. donovani*, *L. infantum*, *L. chagasi*), is a progressive wasting disease of dogs and humans that is often fatal if untreated (*1*). Agents of the *L. donovani* complex occur in parts of Mediterranean Europe, the Middle East, Asia, Africa, and Central and South America (*1–3*). In infections involving the *L. donovani* complex in the Mediterranean region (*L. infantum*) and in South America (*L. chagasi*), dogs are reservoirs for human infection (*1,2*). Parasites are usually transmitted between hosts by phlebotomine sandflies (*Lutzomyia* or *Phlebotomus* spp.) (*2,3*).

Direct quantitative relationships between prevalence of leishmaniasis in local dog populations and incidence of human disease have been reported (*4*). Infection in dogs may indicate human risk for leishmaniasis, especially in HIV-positive persons, in many areas (*5*); infected but asymptomatic dogs can infect sandflies that feed on them, posing a risk to uninfected dogs and humans (*6*).

Until recently, visceral leishmaniasis was thought to be primarily an imported disease in North America; infected dogs had usually been imported from regions in southern Europe or South America where *L. infantum* and *L. chagasi* were enzootic (*2,3*). However, sporadic cases of leishmaniasis have been reported in foxhounds and dogs of other breeds with no history of travel to areas where leishmaniasis was enzootic, and the origin of these infections remains unknown (*7,8*).

In the late summer of 1999, foxhounds at a New York foxhunting club began showing signs of epistaxis, weight loss, muscle atrophy, seizures, alopecia, dermal lesions, swollen limbs and joints, and renal failure (*9*). Of the 250 dogs in the kennel, 112 (44.8%) were sick and 29 (11.6%) had died at the time of the investigation. Cytopathologic examination of joint fluid of 1 hound showed amastigote forms of *Leishmania* spp. These parasites were found at necropsy of several dogs by using indirect immunofluorescent assay (IIF), polymerase chain reaction, culture, and cytologic and histopathologic studies (*9*). At that time, autochthonous leishmaniasis had not been reported in dogs, other animals, or humans in New York.

Diagnostic surveys were initiated to measure the prevalence of *Leishmania* infection at the index hunt club and to determine how infection was introduced into and transmitted among these dogs; the investigation was extended to foxhounds, other breeds of dogs, and wild canids in other states. We describe the results of the 3-year investigation of canine visceral leishmaniasis in the United States and Canada through February 2003.

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Materials and Methods

The Masters of Foxhounds Association of America (MFHA) represents >200 foxhound kennels and hunt clubs that house >12,000 foxhounds in 35 US states and 4 Canadian provinces. In conjunction with MFHA and numerous state health departments, the Centers for Disease Control and Prevention (CDC) invited all MFHA-registered foxhound owners to participate in this investigation. Owners of nonregistered foxhound hunt clubs that were in close proximity to MFHA-registered hunt clubs were also invited to participate. Dog owners were asked to submit samples in 3- to 4-month intervals. From hounds identified as seropositive for *Leishmania* spp., bone marrow and other specimens were requested for parasitologic diagnosis. Serum samples from dogs of breeds other than foxhounds and from wild canids (e.g., foxes and coyotes) were also obtained and tested for antibodies to *Leishmania* spp. and *Trypanosoma cruzi*. Samples from other dog breeds were obtained from kenneled "pound dogs" in Virginia and New York, where infection in foxhounds had been identified, and from pet dogs whose sera were tested at CDC for *Leishmania* antibodies before the pets traveled to countries that require such testing. Fox and coyote samples were provided from animals trapped in various locations of the southeastern United States.

IIF assays for antibodies to *Leishmania* spp. were performed on human and canine serum samples submitted to CDC (*10*). IIF was considered positive when fluorescence was observed around the organisms on the slide. Fourfold dilutions were used to reach the final endpoint titer. The CDC standard IIF diagnostic cutoff titer for infection by *Leishmania* spp. belonging to the donovani complex in dogs is >128 (*10*).

To assess possible infection in humans, persons associated with dogs in the study were invited to submit serum samples for testing. After explaining the purpose of the study and obtaining informed consent, participants were asked about their contact with foxhounds and their personal health status. Serum samples were tested for *Leishmania* antibodies by using the same technique, with the same titer value for determining a positive reaction (*3,10*).

Other antemortem samples submitted from dogs included aspirates of spleen, liver, or lymph nodes and excisional lymph node biopsy specimens. Postmortem specimens submitted included blood, bone marrow, lymph nodes, kidney, spleen, liver, brain, testes, epididymis, ovaries, and neoplasms. Two media were used to culture *Leishmania* spp: Novy-MacNeal-Nicolle (NNN) medium with Offutt modification and modified NNN medium with Roswell Park Memorial Institute medium overlay (*11*).

T. cruzi antibodies cross-react and give false-positive reactions in the CDC *Leishmania* IIF. Because *T. cruzi* is enzootic in domestic dogs and wild canids in some areas of the United States where foxhounds were tested, all samples that yielded *Leishmania* IIF titers >128 were tested in the radioimmunoprecipitation assay (RIPA) for *T. cruzi* at the University of Iowa (*12,13*). Sera that gave positive results in both tests were considered positive for *T. cruzi* infection because *Leishmania* antibodies do not give false-positive reactions in the *T. cruzi* RIPA. A group of sera that yielded *Leishmania* spp. IIF titers <128 were randomly selected from foxhounds that were kenneled in southern states (where *T. cruzi* occurs enzootically in wildlife) to further assess the prevalence of *T. cruzi* infection. We defined a confirmed case of *Leishmania* infection as being culture-positive for *L. infantum*, regardless of antibody titer. A probable case was defined as *Leishmania* IIF titer >128 with a negative RIPA for antibodies to *T. cruzi*.

Selected isolates of *Leishmania* spp. cultured from foxhounds were shipped for subtyping and zymodene analysis to the Istituto Superiore di Sanità in Rome, Italy. Montpelier Centre nomenclature for the identification of agents of human leishmaniases was used to classify the organisms.

Results

From April 2000 to December 2003, >20,000 serum samples were collected from >12,000 canines and submitted to CDC for antibody testing. The dogs ranged in age from 2 months to 13 years. Foxhounds, basset hounds, and beagle hounds represented 91.7%, 2.4%, and 1.3% of the population, respectively. The remainder (4.6%) included >50 other breeds of dogs, foxes, and coyotes.

MFHA-registered fox-hunting clubs are widely dispersed in the eastern half of the United States and Canada, and fewer are located in western states. Of the 210 kennels or hunt clubs that participated in this study, only 29 (14%) were located in states west of the Mississippi River. In contrast, 69 (33%) are located in the 3 states of Pennsylvania, Maryland, and Virginia.

A total of 12,411 dog serum samples from throughout the United States and Canada were submitted to CDC in the first round of sample collection. The distributions of IIF titers in the initial and subsequent rounds of serologic testing are shown in Table 1. Each subsequent round of testing was less comprehensive than the preceding rounds as a result of financial constraints related to collecting blood samples dogs shipping specimens as well as waning interest of owners.

Infection with *Leishmania* spp. was confirmed in foxhounds from 58 hunt clubs or kennels in 18 states and 2 Canadian provinces (Table 2 and Figure 1). The distribution of *T. cruzi*–infected kennels is shown in Table 3 and Figure 2.

Collections of multiple serum specimens from individual hounds during the course of the investigation allowed

Table 1. Distribution of serum *Leishmania* antibody titers in kenneled hunting dogs, United States and Canada, 2000–2003

Serosurvey	IIF titer*					
	16	32	64	128	256	≥512
First serosurvey (n = 12,411)						
Cumulative no. seroreactive	1,667	736	267	190	133	81
Seroprevalence (%)	13.4	5.9	2.2	1.5	1.1	0.7
Second serosurvey (n = 4,614)						
Cumulative no. seroreactive	1,033	511	195	134	99	62
Seroprevalence (%)	22.4	11.1	4.2	2.9	2.1	1.3
Third serosurvey (n = 1,493)						
Cumulative no. seroreactive	438	211	96	67	52	40
Seroprevalence (%)	29.3	14.1	6.4	4.5	3.5	2.7
Fourth serosurvey (n = 792)						
Cumulative no. seroreactive	262	141	79	58	51	38
Seroprevalence (%)	33.1	17.8	10.0	7.3	6.4	4.8
Fifth serosurvey (n = 571)						
Cumulative no. seroreactive	149	91	58	50	42	33
Seroprevalence (%)	26.1	15.9	10.2	8.8	7.4	5.8
Sixth serosurvey (n = 421)						
Cumulative no. seroreactive	115	76	50	42	37	29
Seroprevalence (%)	27.3	18.1	11.9	10.0	8.8	6.9

*IIF, indirect immunofluorescent assay.

detection of seroconversion over time (Table 4). Seroprevalence in each subsequent round of testing was artificially skewed toward higher values as a result of selective and repeated submission of samples drawn from previously seropositive hounds.

Demonstration or isolation of *Leishmania* spp. was attempted on blood and tissue specimens submitted by dog owners or their veterinarians from 185 dogs. Tissue specimens for diagnostic culture were collected and submitted to Walter Reed Army Institute of Research or to CDC. Specimens from 62 (33.5%) of 185 hounds were culture-positive. Unexpectedly, 7 (3.8%) positive cultures were from hounds that had *Leishmania* IIF titers <64 (below the positive cutoff titer).

Isolates from 46 foxhounds were sent to the Reference Centre in Rome, Italy, for zymodeme analysis. The isoenzyme characterization showed the agent isolated from 46 foxhounds to be *Leishmania infantum* zymodeme MON1.

Sera were tested from numerous other breeds of dogs from many states, including shelter dogs from Dutchess County, New York, and Orange County, Virginia, where infection had been confirmed in foxhounds and wild canids (n = 286) collected in the southeastern United States. All of these samples were negative in the *Leishmania* spp. IIF (Table 5). None of the samples from dogs were positive in the *T. cruzi* RIPA; however, *T. cruzi* infection was detected serologically in 2 wild canids.

Serum samples obtained from 158 persons associated with foxhounds were tested by the *Leishmania* spp. IIF. None of the persons who provided blood samples reported signs or symptoms suggestive of leishmaniasis, and all of the samples gave titers below the positive cutoff.

Discussion

Our survey of foxhounds, other breeds of dogs, and wild canids showed that canine visceral leishmaniasis is enzootic in 18 US states and 2 Canadian provinces. Newly seroconverted cases were detected each year during the investigation (2000–2003), which indicates that active transmission of the parasite continues.

Data from this investigation indicate that autochthonous infection in canines is predominantly limited to foxhounds. Increased susceptibility of this breed is possible, although comparative infection studies have not been carried out. Several factors concerning management of foxhounds may favor transmission: the gregarious nature of the breed, concentration of large numbers of dogs within hunt clubs, and management practices, such as intrastate and interstate movement and interbreeding and exchange of dogs between hunt clubs. More than 200 MFHA-registered kennels in the United States and Canada house >12,000 hounds. Most of these dogs are foxhounds that travel frequently and extensively to participate in field trials, for trading and interbreeding between kennels and hunt clubs, and to be shown at dog shows. These management practices appear to have enabled leishmanial infection to spread widely in this breed throughout the eastern United States and Canada.

Testing for *Leishmania* antibodies in pet dogs from numerous states and in shelter dogs in New York and Virginia failed to identify any *Leishmania*-positive animals. Similarly, no antibody evidence of *Leishmania* infection was detected among 291 wild canids taken from various states where infections in kenneled foxhounds were diagnosed. By contrast, 2 wild canids were infected with *T. cruzi*. Taken together, these findings suggest that

Table 2. Distribution of participating foxhound hunt clubs or kennels showing number of hunt clubs with hounds infected with *Leishmania* spp.

State or province	Total hunt clubs tested/ positive hunt clubs* (%)
Alabama	4/2 (50)
Arkansas	1/0
Arizona	1/0
British Columbia	1/0
California	4/0
Colorado	3/0
Connecticut	3/2 (66.7)
Florida	6/0
Georgia	6/1 (16.7)
Iowa	2/1 (50)
Illinois	8/4 (50)
Indiana	2/1 (50)
Kansas	2/0
Kentucky	5/2 (40)
Maryland	17/3 (17.6)
Massachusetts	4/0
Michigan	3/2 (66.7)
Minnesota	1/0
Mississippi	2/0
Missouri	3/1 (33.3)
Montreal	1/0
North Carolina	10/3 (30.0)
Nebraska	2/0
Nevada	1/0
New Hampshire	2/0
New Jersey	5/1 (20)
New Mexico	2/0
New York	10/1 (10)
Nova Scotia	1/1
Ohio	7/3
Oklahoma	11/0
Ontario	9/5 (55.6)
Pennsylvania	22/3 (13.6)
South Carolina	7/1 (14.3)
Tennessee	9/1 (11.1)
Texas	9/0
Virginia	32/12 (37.5)
Vermont	1/0
Washington	1/0

*Positive hunt clubs are defined as those that contained $\geq$1 positive dog.

Leishmania infection in foxhounds is transmitted from dog to dog.

Antibody testing of 158 humans associated with infected foxhounds did not identify any seropositive persons, nor have autochthonous cases of visceral leishmaniasis in humans been diagnosed in North America. Transmission among foxhounds and other breeds appears to be limited to direct dog-to-dog mechanisms; this assumption is supported by lack of apparent transmission to humans. The fact remains, however, that a zoonotic disease has been introduced into the canine population in the United States and Canada. Visceral leishmaniasis in humans has variable onset and manifestations, and delay or misdiagnosis in areas where the disease is not endemic is common (*14*).

In regions of transmission outside North America, canine visceral leishmaniasis, caused by *L. infantum* (*L. chagasi* in the New World), is transmitted by sandfly vectors (*1–3*). Although sandflies indigenous to North America have not been implicated in transmission of visceral leishmaniasis, 4 species of North American sandflies of the genus *Lutzomyia* are mammalian feeders. *Lutzomyia anthorphora* and *Lu. diabolica* are found in Texas, and *Lu. cruciata* is found in Florida and Georgia (*15*). *Lu. shannoni* has been identified in Alabama, Arkansas, Delaware, Florida, Georgia, Louisiana, Mississippi, North Carolina, South Carolina, and New Jersey (*15*). The range of *Lu. shannoni* overlaps the locations of many of the hunt clubs in which we found *Leishmania*-infected dogs.

Experimental studies showed that *Lu. shannoni* became infected with *L. infantum* after feeding on *L. infantum*–infected dogs (*16*). Investigators hypothesized that these insects were competent vectors and could initiate new enzootic cycles of *Leishmania* transmission in areas where infected animals were introduced (e.g., North America) (*16*). As the reservoir of infection in canine hosts becomes larger and more dispersed, the possibility increases that conditions will lead to exposure of competent vectors and subsequent vectorborne transmission.

The data collected in this investigation and the apparent absence of active vector transmission suggest that spread of infection in foxhounds and other dogs occurred by direct dog-to-dog transmission. Infected dogs, including those in preclinical or subclinical stages, can be reservoirs of infection for uninfected animals. Possible modes of direct dog-to-dog transmission include biting, reusing needles for injections, blood transfusions, and breeding. Dog bites and other abrasions and lacerations occur commonly

Figure 1. Distribution of hunt clubs with confirmed cases of visceral leishmaniasis, United States and Canada. States in which hunt clubs or kennels had $\geq$1 dog infected with *Leishmania infantum* are shaded. *Leishmania*-positive foxhounds were also found in Nova Scotia and Ontario.

Table 3. Distribution of participating foxhound hunt clubs or kennels showing number of hunt clubs with hounds infected with *Trypanosoma cruzi*.

State or province	Total hunt clubs tested/ positive hunt clubs* (%)
Alabama	4/2 (50)
Arkansas	1/1 (100)
Arizona	1/0
British Columbia	1/0
California	4/0
Colorado	3/0
Connecticut	3/0
Florida	6/3 (50)
Georgia	6/1 (16.7)
Iowa	2/0
Illinois	8/0
Indiana	2/0
Kansas	2/1 (50)
Kentucky	5/0
Maryland	17/3 (17.6)
Massachusetts	4/0
Michigan	3/0
Minnesota	1/0
Mississippi	2/0
Missouri	3/1 (33.3)
Montreal	1/0
North Carolina	10/2 (20)
Nebraska	2/0
Nevada	1/0
New Hampshire	2/0
New Jersey	5/0
New Mexico	2/0
New York	10/0
Nova Scotia	1/0
Ohio	7/1 (14.3)
Oklahoma	2/1 (50)
Ontario	9/1 (11.1)
Pennsylvania	22/0
South Carolina	7/2 (28.6)
Tennessee	9/3 (33.3)
Texas	9/0
Virginia	32/4 (12.5)
Vermont	1/0
Washington	1/0

*Positive hunt clubs are defined as those that contained ≥1 positive dog.

among working and kenneled foxhounds, which may potentiate exchange of body fluids between hounds. Blood transfusion transmission from infected dogs was documented in a clinical study at the University of Pennsylvania (*17*). Congenital transmission from infected, pregnant female foxhounds to their pups was observed by owners and reported during the course of our investigation. Transplacental transmission in an experimentally infected beagle was recently described by Rosypal et al. (*18*). Breeding that results in transplacental infection of litters may be the most important mechanism of transmission among foxhounds, which explains why this infection is limited to foxhounds even in situations in which foxhounds are housed with beagles and basset hounds (data not shown).

Cross-reacting anti–*T. cruzi* antibodies give false-positive results in the CDC *Leishmania* IIF, but anti-*Leishmania* antibodies do not give false-positive results in the *T. cruzi* RIPA (*12,13*). We took advantage of this difference by doing RIPA testing on samples from 413 hounds that had *Leishmania* IIF titers ≥32. Eighty-six (21%) of these specimens gave positive results, which indicates that the hounds were infected with *T. cruzi*. The remaining 326 (78.9%) that were negative by RIPA were considered to be infected with *L. infantum*. Dogs with dual reactivity to the *Leishmania* and *T. cruzi* antibody assays may have been infected with both protozoal agents; however, we could not confirm this possibility, and we believe that the probability is low and inconsequential to this study.

Previous reports across several decades have indicated that *T. cruzi* is enzootic in domestic dogs and wild canids in the southern United States (*12,13*), a consequence of the sylvatic cycle of *T. cruzi* that involves triatomine insects and various mammalian hosts. Dogs are believed to become infected by exposure to infected vectors or by eating infected wild mammals, such as armadillos, raccoons, opossums, and wood rats. In contrast to the situation with *Leishmania* spp., direct dog-to-dog transmission of *T. cruzi* is likely less frequent, although congenital transmission and transmission through blood transfusion may help maintain the parasite in dog populations.

The widespread geographic distribution and prevalence of *T. cruzi* infection in hounds reported here expand our understanding of this highly pathogenic parasite in dogs in the United States. *T. cruzi* causes severe clinical manifestations in dogs (*13*), as it does in humans (*19*). In view of these findings, veterinarians in enzootic areas should include *T. cruzi* infection in the differential diagnosis of dogs with unexplained cardiac disease. Unfortunately,

Figure 2. Distribution of hunt clubs with *Trypanosoma cruzi*–positive hounds, United States and Canada. States in which hunt clubs or kennels had ≥1 dog infected with *T. cruzi* are shaded. A *T. cruzi*–positive hunt club was also found in Ontario.

Table 4. Positive seroconversion to *Leishmania* spp. or *Trypanosoma cruzi* in kenneled hunting dogs, United States and Canada, 2000–2003*

Characteristic	2000	2001	2002	2003
No. samples tested	12,446	5,487	1,208	1,306
No. new *Leishmania*-positive samples	33	49	9	2
No. new *T. cruzi*–positive samples	6	14	1	0

*Numbers do not include hounds surveyed at the index hunt club in New York.

transmission of *T. cruzi* to dogs cannot be prevented other than by effective vector control and not allowing dogs to run unsupervised, and no curative treatment for *T. cruzi* infection is available.

Participation in this investigation by foxhound owners was voluntary, and the loss to follow-up of many animals after the initial serosurvey detracted from our ability to comprehensively assess the incidence and geographic extent of this infection. Although new cases of leishmaniasis and trypanosomiasis were discovered in the sequential serosurveys, we could not calculate the incidence of these parasitoses among exposed foxhounds. As a result of decreasing compliance, the second and subsequent serosurveys were less comprehensive than the initial serosurvey, despite our request that all hounds be retested. Although serologic testing at CDC was offered at no charge, decreasing participation may have been caused by the cost of specimen collection and shipment or by declining interest.

The distribution of canine visceral leishmaniasis in the United States and Canada was determined by using defined case definitions based on confirmatory laboratory data. Data were analyzed with strict adherence to case definitions. Twelve suspected cases were excluded from analysis because they did not meet the case definitions.

Preliminary recommendations to limit spread of *Leishmania* infection were communicated to foxhound owners in 2000. CDC recommended a moratorium on exchange of hounds between hunt clubs and commingling of hounds from different hunt clubs for at least 1 year. Intra-hunt club recommendations to limit transmission were also suggested.

Recommendations to segregate infected animals, suspend dog shows and hunting for clubs or kennels with dogs with leishmaniasis, and avoid commingling and interbreeding of animals between hunt clubs were implemented initially by most of the hunt clubs or kennels. Nevertheless, cooperation appears to have been short-lived. In 2001, after 1 year of general adherence to the recommendations, hunting and commingling resumed and continues in most hunt clubs and kennels. Factors that led to this decision in the hunting community included the belief that the disease was not a threat to the well-being of the animals or the persons involved. The perceived low illness and death rates associated with leishmaniasis in this canine population and the inconvenience and cost of recommended control measures may have also led some hunt club owners to ignore them.

The presence of *L. infantum*–infected dogs in areas in the United States and Canada where sandflies have not been identified is now well established. Sandflies may exist in these areas but have not yet been identified, or another arthropod species may be responsible for *Leishmania* transmission. The mechanisms by which canine visceral leishmaniasis can be transmitted among dogs in the absence of vectors warrant further investigation. Because most leishmanial infections in dogs appear limited to foxhounds, breed susceptibility and breeding-associated transmission mechanisms should be further examined.

The widespread presence of *L. infantum* infection in foxhounds in North America represents potential public and canine health threats that should be addressed by further investigation and control measures. Based on findings from this investigation and what is known about the biologic behavior of *Leishmania* spp., the following recommendations should be considered to manage and control leishmaniasis in foxhound kennels. All dogs in a hunt club or kennel should be tested serologically to identify those infected with the parasite. All dogs considered for breeding

Table 5. Distribution of *Leishmania* antibody titers in pet dogs, shelter dogs, and wild canids, 2001–2002

	Titer						
Animal	<16	16	32	64	128	256	>512
Pet dogs* (n = 709)	706	3	0	0	0	0	0
Shelter dogs,† Dutchess County (n = 74)	71	2	1	0	0	0	0
Shelter dogs,† Orange County (n = 55)	53	2	0	0	0	0	0
Wild canids‡ (n = 291)	286	2	2§	0	1§	0	0

*Samples from pet and sporting dogs submitted to the Centers for Disease Control and Prevention from numerous states for leishmaniasis testing to fulfill entry requirements for countries that require it.
†Samples from dogs retained in county animal shelters.
‡Samples from wild canids collected in the southeastern United States included. Species included red fox (*Vulpes vulpes*, n = 158), gray fox (*Urocyon cinereoargentus*, n = 51), and coyote (*Canis latrans* , n = 82).
§1 of the 2 canids with titer of 32 and the canid with titer of 128 were *Trypanosoma cruzi* RIPA-positive.

should be >2 years of age and should be evaluated serologically for *Leishmania* infection. Dogs with confirmed or suspected leishmaniasis should be excluded from breeding programs. Dogs with positive serum *Leishmania* titers should have cultures performed to confirm infection status. All dogs that are confirmed to have *Leishmania* infection should be euthanized. Dogs with borderline or suspicious titers should be considered as possibly infected and retested in 3–6 months for further assessment. All dogs that have positive results when tested for *Leishmania* antibodies and have either not had specimens cultured or had culture-negative results should be tested for specific antibodies to *T. cruzi*. The recent report of an effective vaccine for canine leishmaniasis (*20*) suggests an additional tool to prevent and eliminate this infection in North America, although further research is necessary to define its role in a prevention strategy. The effectiveness of control measures must be monitored by surveillance of foxhounds and associated dog breeds by using sensitive diagnostic screening methods.

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Chemoprophylaxis and Malaria Death Rates

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To determine the effect of chemoprophylaxis on the case-fatality rate of malaria, we analyzed all cases of *Plasmodium falciparum* malaria in nonimmune persons reported from 1993 to 2004 in Germany. In univariate and multivariate logistic regression analysis, we determined the effect of age, sex, chemoprophylaxis, chemoprophylactic regimen, compliance for chemoprophylactic regimen, exposure prophylaxis, country of infection, and year of reporting on the outcome. Of 3,935 case-patients, 116 (3%) died of malaria. Univariate analysis showed significant associations with death for chemoprophylaxis with chloroquine plus proguanil compared to no chemoprophylaxis. The multivariate model showed that patients who had taken chemoprophylaxis were less likely to die compared to those who had not taken chemoprophylaxis, adjusted for patient age and reporting year. The study demonstrated that chemoprophylaxis significantly reduced fatality rates among nonimmune malaria patients and supports the importance of existing guidelines for malaria prevention.

The estimated risk of nonimmune travelers to malaria-endemic countries acquiring malaria is 1–357 per 100,000 depending on endemicity of the country (*1*). Approximately 800 imported malaria cases are reported through the notifiable disease surveillance system in Germany each year, about twice as many per population as in the United States (*2*). Within the World Health Organization European Region, Germany is the country with the third largest number of reported imported malaria cases following France and the United Kingdom (*3*).

Exposure prophylaxis (repellents and bed nets) and chemoprophylaxis are established methods of preventing malaria during travel in malaria-endemic countries; its importance has recently been underlined by Chen and Keystone as well as by Zuckerman (*4,5*). Persons from nonendemic countries are considered nonimmune because their risk of acquiring malaria and subsequently developing severe disease with possible fatal outcome is considerably higher than for adults who have spent their childhood in a malaria-endemic environment (*6*).

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The lack of randomized controlled trials on the effectiveness of chemoprophylaxis on appropriately characterized travelers has been rightly criticized, yet the methodologic difficulties of realizing such investigations are obvious (*7*). Alternative study designs based on surveillance data may provide some evidence whether nonimmune travelers to malaria-endemic countries would have benefited from chemoprophylaxis even if it had failed to prevent the disease. The strongest outcome measure for this question is the case-fatality ratio (CFR). Because fatal malaria is rare in nonendemic countries, various studies on imported malaria have not had the statistical power to investigate the case-fatality rate under inclusion of relevant confounders (*8,9*). Multinational networks able to overcome the problem of small sample size collect their data from specialized centers, causing a number of selection biases that may have particular impact on the CFR (*8,10*).

Methods

From 1993 to 2000, physicians and laboratories in Germany reported malaria cases to local health departments, which then sent special case report forms to the Robert Koch Institute, the federal agency for infectious disease control. The forms contain information on age, sex, travel history, *Plasmodium* species, prophylactic measures, onset of disease, and death. Since 2001, after new legal requirements, laboratories and physicians report directly to the Robert Koch Institute. The report forms have had only minor changes over the years, which ensures comparability of the data.

The study was limited to reported *Plasmodium falciparum* malaria in persons from nonendemic countries. A case of *P. falciparum* malaria was determined when *P. falciparum* was directly detected in a person's blood. All cases of mixed infections containing *P. falciparum* and another subspecies were removed from the analysis.

Endemicity of a country was determined by using the World Health Organization's list of malaria-endemic countries (*11*). Only persons with German nationality or origin

or cases originating from other nonendemic countries were considered nonimmune and included in the study. Country of infection was defined as the malaria-endemic country in which the patient stayed during the incubation period. If >1 country was named, the region or continent to which all countries belong was used.

Death was used as the outcome variable. The following confounding variables were considered for the analysis: age, sex, year of reporting, chemoprophylaxis, chemoprophylactic regimen, patient compliance for chemoprophylaxis, exposure prophylaxis (repellents and bed nets), and country of infection. All but the first 3 variables were assessed by patient history. Information on type of treatment and time between onset of symptoms and treatment was not included in the analysis as it was not consistently available throughout the study period.

For univariate and multivariate logistic regression analysis, we used SPSS version 13.0 (SPPS Inc., Chicago, IL, USA). The method for variable selection was forward stepwise (using likelihood ratio statistics) taking into account all variables listed in Table 1. The confidence interval (CI) for all analysis was 95%.

Results

From 1993 to 2004, the Robert Koch Institute received reports on 6,964 cases of *P. falciparum* malaria, 2,371 cases due to other species or mixed infections, and 521 cases due to unidentified species. Among the cases of *P. falciparum* malaria, 3,935 (57%) patients were nonimmune and included in the subsequent analysis. A total of 116 patients in this study population died, resulting in a CFR of 3% (Table 2). Chemoprophylaxis was taken by 1,581 (42%) of the 3,752 persons for whom this information was available. The proportion of persons who took chemoprophylaxis declined over the years (Figure). Univariate analysis of risk factors is shown in Table 1. Variables not shown in these tables were not significantly associated with death in any of the analyses.

Univariate analysis showed that increasing age and infection acquired in Africa were positively associated with fatal outcome. Chloroquine plus proguanil was inversely associated with fatal outcome compared to no chemoprophylaxis. The year of reporting was significantly associated with fatal outcome but did not show a linear association.

The results of multivariate analysis are shown in Table 3. In contrast to the univariate analysis, chemoprophylaxis was significantly associated with death. Age and reporting year remained significantly associated in the multivariate model (Table 3).

Discussion

This study demonstrated an independent effect of chemoprophylaxis on fatal outcome. For nonimmune

Table 1. Univariate analysis of risk factors for fatal outcome of imported *Plasmodium falciparum* malaria in nonimmune patients, Germany 1993–2004

Risk factor	Odds ratio	95% confidence interval	p value
Chemoprophylaxis, total (n = 3,752)			
No	1		
Yes	0.743	0.493–1.121	0.157
Chemoprophylaxis, comparison of regimens (n = 3,752)			0.047
None (n = 2,171)	1		
Chloroquine alone (n = 485)	1.372	0.824–2.285	0.225
Proguanil alone (n = 59)	0.550	0.075–4.030	0.556
Mefloquine alone (n = 322)	0.503	0.201–1.258	0.142
Chloroquine plus proguanil (n = 459)	0.280	0.102–0.773	0.014
Other (n = 256)*	0.765	0.328–1.784	0.536
Compliance of chemoprophylaxis (n = 3717)			0.293†
No chemoprophylaxis	1		
Chemoprophylaxis with incomplete or unknown compliance	0.829	0.535–1.284	0.401
Chemoprophylaxis complete	0.538	0.231–1.249	0.149
Age (n = 3,844)			
Continuously (by year)	1.055	1.040–1.070	<0.001
Sex (n = 3,901)			
Female	1		
Male	1.141	0.768–1.695	0.515
Country of infection in Africa (n = 3,832)			
No	1		
Yes	3.642	1.150–11.529	0.028
Reporting year (n = 3,935)			0.004†

*235 were combinations of drugs that are not officially recommended regimens, 15 were doxycycline alone, and 6 were atovaquone/proguanil.
†Overall p value for the categoric variable.

Table 2. Imported *Plasmodium falciparum* malaria among nonimmune persons in Germany, 1993–2004

Year	No. cases	No. deaths (%)
1993	258	5 (1.94)
1994	419	19 (4.53)
1995	349	15 (4.30)
1996	412	13 (3.16)
1997	406	9 (2.22)
1998	378	19 (5.03)
1999	428	20 (4.67)
2000	326	2 (0.61)
2001	312	7 (2.24)
2002	232	2 (0.86)
2003	227	3 (1.32)
2004	188	2 (1.06)
Total	3,935	116 (2.95)

patients with *P. falciparum* malaria who had taken any chemoprophylaxis (adjusted for age and reporting year), the risk of dying of the disease was two thirds that of those who had not taken any chemoprophylaxis (odds ratio [OR] 0.63, 95% CI 0.40–0.98). We are not aware of any such association being reported. Yet the findings are consistent with earlier reports (*9,12*). Our findings are also in line with observations made in numerous case reviews in which severity of illness appeared to be lower among patients who had taken chemoprophylaxis compared to patients who had not (*13–15*). Fatal outcome could be seen as the consequence of severe malaria, which in turn is associated with high parasitemia (*9,13*). If unable to prevent infection, chemoprophylaxis would likely slow down the parasite growth rate, which would result in a larger window of opportunity in which treatment might prevent death (*14,16*). Our data suggest that even in cases where chemoprophylaxis fails to prevent the development of malaria, it still significantly reduces the risk of dying from it. This finding may be important for travelers to malaria-endemic countries, adding another good reason to take chemoprophylaxis, in addition to reducing the risk of acquiring the disease.

Our study was also able to individually analyze specific chemoprophylactic regimens and identify significant associations for some of the individual regimens. In the univariate analysis, the risk of dying from malaria for patients who had taken the combination of chloroquine plus proguanil as a chemoprophylaxis regimen was less than one third that of those patients who had not taken any chemoprophylaxis (OR 0.28, 95% CI 0.10–0.77).

Chemoprophylaxis with doxycycline, atovaquone/proguanil, mefloquine, or proguanil did not show a significant association. This finding may be because the smaller prevalence of these regimens may have resulted in insufficient statistical power and does not necessarily question the prophylactic effectiveness of these regimens (*7*).

We can assume that recommendations for chemoprophylaxis were quite similar at any given point in time, since our study population was limited to Germany, and they agree with the current recommendations in the United States and the United Kingdom (*5,17–21*). The risk for infection, particularly the prevalence of chloroquine-resistant *P. falciparum*, has changed over the years in some endemic regions, and our study design has partly controlled for this by including the reporting year into the model.

The analysis also showed that increasing age was an independent risk factor for death. Age has been identified as a risk factor for severe disease or fatal outcome of malaria in several studies and case reports from the United States, Europe, and Israel (*8,9,12,15,16,22,23*). In contrast to those previous studies, we decided not to group the age into categories because our study population was sufficiently large to use age as a continuous variable, which allowed us to avoid any kind of classification bias. Our findings confirmed that the risk of dying from malaria increases with age (OR 1.06, 95% CI 1.04–1.07). As discussed by Mühlberger et al., the most likely explanation for the observed age effect is that with increasing age the immune system looses its capacity to generate a competent immune response against previously unencountered pathogens (*12*). Our study adds an important conclusion to this finding: although the elderly have an increased risk of dying from malaria, they can significantly reduce this risk by taking chemoprophylaxis.

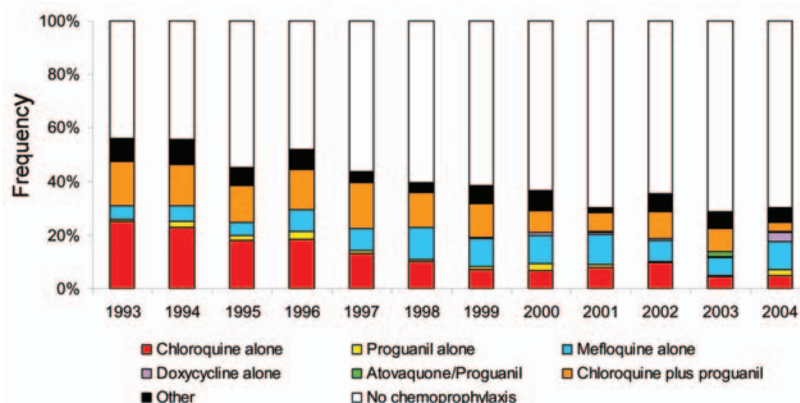

Figure. Proportional frequency of chemoprophylactic regimen taken by nonimmune patients with imported *Plasmodium falciparum* malaria, Germany, 1993–2004.

Table 3. Multivariate analysis of risk factors for fatal outcome of imported *Plasmodium falciparum* malaria in nonimmune patients, Germany 1993–2004

Risk factors (N = 3,681)	Odds ratio	95% confidence interval	p value
Chemoprophylaxis			
No	1		
Yes	0.629	0.403–0.983	0.042
Age	1.055	1.039–1.070	<0.001
Reporting year			0.003*

*Overall p value for the categoric variable.

The reporting year proved to be significantly associated with the CFR. We controlled for it by including it in the model as a categoric variable, since the association was not a linear one. We recommend that controlling for the year of data collection should also be considered in similar analyses of data collected over an extended period of time.

Although a technical change in the reporting mechanism occurred in 2001, it is not likely associated with the observed change of CFR; the decline in CFR was already observed before 2001, and the national death registry also showed a parallel decline of malaria deaths (*24*). From 1989 to 1995, CFR for all cases has generally been higher in Germany (3.6%) than in several other European countries and the United States (≈1%) (*25*). Meanwhile, CFR in Germany has declined to <1%. This decline may have been caused by a combination of better prophylactic regimens, improved pretravel counseling, chemoprophylaxis compliance, and earlier diagnosis and treatment. The treatment delay and type of treatment, in particular, might have played a role. Although this information was not consistently available in the study population, reporting forms have been changed so that future analyses should provide some evidence for or against this hypothesis. Additionally, physicians and pharmacies have begun providing pretravel advice, which may have affected the aforementioned factors. Providing this advice in the past has been the domain of a few highly specialized centers (*26*).

The study was focused on nonimmune patients, which were identified by their nationality or citizenship (until reporting year 2000) or by country of origin (from reporting year 2001 onwards). Both variables serve as a proxy for non-immunity and have been used as such in previous studies (*12,15*). During a transition period from 1999 to 2000, nationality and country of birth were simultaneously assessed in our surveillance system, and a comparison of both variables showed that the discrepancy was ≈5%. Therefore, we do not expect this technical change to have any relevant impact on our findings. Legal constraints do not allow collecting information on ethnicity or more detailed information on the geographic origin of a person in Germany.

In contrast to studies based on single institutions or networks of specialized centers, our study population is representative in that it included cases identified by any laboratory regardless of where and how the patient was treated. This strategy reduces the risk for selection bias, which is of particular importance when studying CFR.

The univariate analysis indicates that malaria acquired in Africa has a higher CFR than malaria acquired elsewhere (*10,13*). Lewis et al. have shown that severe malaria was observed more commonly in patients returning from countries in central, southern, and eastern Africa compared to those returning from countries in western Africa (*15*). However, risk assessment with reference to the country of infection is problematic, as reliable denominator data on exposure are difficult to obtain, often do not take the duration of exposure into account, and may not be reliable (*25,27–30*).

While chemoprophylaxis clearly reduces the risk of acquiring malaria in nonimmune persons, the travelers' compliance in taking chemoprophylaxis is quite variable (*3,30–32*). Depending on the country and the method of assessment, the proportion of malaria patients who take chemoprophylaxis is 19%–90% and has repeatedly been identified as a major limitation of preventing imported malaria (*2,4,14,33,34*). Like the recent publication by Askling et al. (*1*), this work demonstrates how data originating from notifiable disease surveillance may lead to research results with important clinical implications, therefore underlining the importance of such surveillance systems. We demonstrated that chemoprophylaxis significantly increases the chance of nonimmune patients to survive imported *P. falciparum* malaria. We suggest that this information be used in pretravel counseling to further motivate persons traveling in malaria-endemic countries to comply with recommended chemoprophylactic regimens.

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Self-medication with Antimicrobial Drugs in Europe

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We surveyed the populations of 19 European countries to compare the prevalence of antimicrobial drug self-medication in the previous 12 months and intended self-medication and storage and to identify the associated demographic characteristics. By using a multistage sampling design, 1,000–3,000 adults in each country were randomly selected. The prevalence of actual self-medication varied from 1 to 210 per 1,000 and intended self-medication from 73 to 449 per 1,000; both rates were high in eastern and southern Europe and low in northern and western Europe. The most common reasons for self-medication were throat symptoms (e.g., dry, inflamed, red, or sore throat, inflamed tonsils, tonsil pain). The main medication sources were pharmacies and medication leftover from previous prescriptions. Younger age, higher education, and presence of a chronic disease were associated with higher rates of self-medication. Attempts to reduce inappropriate self-medication should target prescribers, pharmacists, and the general public.

Antimicrobial drug resistance is a rapidly increasing global problem (*1,2*), and prevalence varying widely among countries (*3*). Prevalence of resistance is positively correlated with prescribed outpatient drug use on a national level (*4,5*). However, actual consumption of drugs may also include self-medication, i.e., using drugs obtained without prescription. Other sources of self-medication may include leftover drugs from treatment courses prescribed earlier or drugs obtained from relatives or friends. Use without medical guidance is inappropriate because using insufficient dosages or incorrect or unnecessary drugs increases the risk of the selection of resistant bacteria (*6*) and the spread of antimicrobial drug resistance (*7*).

To date, the information on self-medication with antimicrobial drugs in the industrialized world is limited. In the United States, several studies indicate considerable use of leftovers (*8–10*), drugs obtained from a family member, a pharmacy, or a source outside the country (*11,12*). For example, in an Hispanic neighborhood of New York City, antimicrobial drugs are available without a prescription (*13*). In Europe, studies describing self-medication and storage of antimicrobial drugs in Spain (*14,15*), Greece (*16,17*), Russia (*18*), and Malta (*19*) also suggest considerable use of the drugs without consulting a physician. However, these studies were small or used selected samples and were not carried out in northern and western Europe. Moreover, because of the different research methods used, no meaningful comparison between countries was possible. In addition, little information exists on factors that puts person at-risk for self-medication. This survey was designed to fill that gap for 19 European countries: Austria, the Netherlands, Sweden, United Kingdom, Ireland, Denmark, Italy, Malta, Luxembourg, Belgium, Spain, Israel, Romania, Czech Republic, Slovakia, Lithuania, Slovenia, Croatia, and Poland. The aim of this study was to estimate and compare the prevalence of actual self-medication and at-risk for self-medication with antimicrobial drugs in participating countries. The demographic characteristics associated with such use,

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the types of drugs used, the sources of self-medication, the symptoms for which the drugs were reportedly used, and duration of use were also examined.

Methods

Countries participating in the study were recruited from 2 networks of surveillance systems: European Surveillance of Antimicrobial Consumption (*20*) and European Antimicrobial Resistance Surveillance System (*21*). A multistage sampling design was used for sample selection in each participating country. Within each country, a region with average prescribed antimicrobial drug consumption was chosen. In those countries where this information was not available (Poland, Czech Republic, Lithuania, Croatia, and Romania), a region was selected that was representative of the country's population in terms of age and sex. In each region, a city (75,000–750,000 inhabitants) and a rural area (5,000–10,000) were selected. By using population registries, including lists of persons in the identified cities and rural areas, persons ≥18 years of age were selected by simple random sampling (computer-generated random numbers). To calculate the sample size, we needed the standard deviation of the unknown prevalence. As the standard deviation was a function of this unknown prevalence, we took the maximal possible value of the standard deviation (*22*). To obtain a precision of 0.05, the sample size needed was 400 persons per country. To adjust for possible nonresponse, we selected larger samples; sample sizes in the countries were 1,000–3,000 persons, equally distributed in urban and rural areas. Self-administered questionnaires were mailed between March and July 2003, and reminders with a new questionnaire attached were sent 2–4 weeks later.

We developed an English questionnaire specifically for this survey, translated it into national languages, and back-translated it to ensure consistency.[1] The questionnaire was pilot-tested in each country. It could be completed either anonymously or with identifiable details to allow a follow-up study. Questions asked about the respondent's use of antimicrobial drugs during the past 12 months, how they were obtained, how they were stored at home, and whether the respondent would consider using drugs without consulting a physician. Details of the drugs used (name of the medicine, symptom or disease coded with International Classification of Primary Care codes [*23*], and duration of use) and demographic characteristics of the respondents were included. Antibacterial drugs for systemic use (Anatomical Therapeutic Chemical class J01) (*24*) were included in the analyses. Medicines erroneously reported as antimicrobial drugs were excluded from the analyses. Ethics or data committee approval for the survey was required in Sweden, Denmark, Belgium, United Kingdom, Ireland, Malta, Czech Republic, Slovenia, Croatia, Romania, and Lithuania and was obtained from the local ethics or data committees of these countries.

Respondents were classified as self-medicating if they reported that they had taken any antimicrobial drugs in the previous 12 months without a prescription from a physician, dentist, or nurse and as prescribed users if antimicrobial drugs had been prescribed. (Physician respondents who reported using nonprescribed drugs were not classified as self-medicating.) Those classified as at-risk for self-medication included those who indicated the intention to self-medicate or store drugs at home. Intended self-medication was defined as answering "yes" or "maybe" to the question, "In general, would you use antimicrobial drugs for yourself without contacting a doctor/nurse/hospital?" Two estimates were used to assess storage of drugs: a maximum estimate, including all respondents who stored antimicrobial drugs, and a conservative estimate that excluded those respondents who stored antimicrobial drugs and had taken the same drugs for a prescribed course in the previous 12 months.

Statistical Analyses

Descriptive statistics were used to estimate the prevalence rates per 1,000 respondents and 95% confidence intervals (CI) for actual self-medication and prescribed use in the previous 12 months and for at-risk self-medication in each country. To assess possible bias from low response rates, we also estimated adjusted prevalence rates. We applied the continuum of the resistance model (*25*), based on the assumption that late respondents most resemble nonrespondents. Late respondents in our study were those who replied after the reminder. The adjusted prevalence is considered similar to the observed prevalence when it falls in the 95% CI of the observed prevalence.

The effects of individual characteristics and country on antimicrobial drug self-medication were studied with logistic regression analyses by using 3 outcome variables: actual self-medication in the previous 12 months, storage of antimicrobial drugs, and intended self-medication. Countries were grouped together in 3 European regions: northern and western (Sweden, Denmark, the Netherlands, Austria, Belgium, Luxemburg, United Kingdom, and Ireland), southern (Malta, Italy, Israel, and Spain), and eastern (Czech Republic, Slovenia, Croatia, Poland, Slovakia, Romania, and Lithuania). This grouping was based on patterns of prescribed use of antimicrobial drugs (*4*), geographic location (*26*), similarities in healthcare systems, and socioeconomic development. The former socialist countries are referred to as eastern countries. We tested possible interactions between the factors found to be

[1]A copy of the questionnaire is available from the corresponding author on request.

significant and set the significance at $p \leq 0.01$ for interaction terms due to multiple testing. Multivariate logistic regression was also used to study the relationship between intended self-medication, storage, and actual self-medication in the previous 12 months. Data were analyzed by using SPSS (version 11) for Windows (SPSS Inc, Chicago, IL, USA).

Results

A total of 15,548 respondents completed the questionnaires. The mean response rate of the countries was 40% (Table 1).

Prevalence of Self-medication and Prescribed Use

The prevalence rates of antimicrobial drug self-medication (actual and at-risk) and prescribed use are presented separately for countries with response rates ≥40% and <40% (Table 2). In both of these groups, prevalence rates for actual self-medication were highest in eastern (in particular, Romania and Lithuania), followed by rates in southern (Malta, Spain, and Italy) Europe. The lowest rates were in northern and western (the Netherlands and Sweden) Europe. The rates of at-risk self-medication also tended to be higher in southern and eastern Europe than in northern and western Europe. The adjusted estimates of prevalence rates of self-medication were similar to the observed rates for many of the countries.[2] In Luxembourg, Austria, Israel, Spain, and Lithuania, the adjusted rates of self-medication were consistently higher than the observed rates, indicating that the observed rates may underestimate the prevalence in these countries. By contrast, in Romania, Croatia, and Slovenia, the adjusted rates were lower than the observed rates, indicating that the prevalence rates might be overestimated in our study.

We compared our estimates of antimicrobial drug self-medication with data available from the European Union's Eurobarometer survey in October 2002 (*27*). We calculated the prevalence of drug use from "leftovers" and drugs "directly from the pharmacy" by using the Eurobarometer data and compared these figures with the same estimates in our study. The estimates were similar with overlapping 95% CIs (data not shown) for countries with both high and low response rates. Our figures differed regarding Spain, for which we found a higher prevalence of self-medication than did the Eurobarometer. Three other studies (*4,15,28*) indicated an even higher prevalence of self-medication in Spain than in our estimate.

Types of Antimicrobial Drugs Used for Self-medication and Duration of Use

Antimicrobial drugs from all classes were used for self-medication in countries with response rates both ≥40% and <40% (information is shown in the online Appendix Figure, available at http://www.cdc.gov/ncidod/eid/vol12no03/05-0992_app-G.htm). Penicillins were the most commonly used, representing 54% of total courses in all countries. Among the countries with response rate >40%, southern and eastern countries used significantly more broad-spectrum penicillin for self-medication than northern and western countries (χ^2, $p<0.05$). This difference was significant when the analysis was repeated and included all countries (χ^2, $p<0.01$). Seventeen courses of self-medication with chloramphenicol in Lithuania and 1 course in the Czech Republic were found (data not shown). Ten courses of self-medication with parenteral (injectable) antimicrobial drugs, namely streptomycin or gentamicin, were found in Lithuania (data not shown). The median duration of actual self-medication was 5 days (1 to 100 days) and was significantly longer among the respondents who had chronic diseases (Mann-Whitney U test, $p<0.01$).

Reasons for Self-medication and Sources

A throat symptom (including red or sore throat), teeth or gum symptoms, and bronchitis were the most common reasons for self-medicating (Figure 1). Eye infection, pain, prostatitis, urogenital infection, headache, and "bad health" were among the other reasons for self-medication (data not shown). In countries with response rates >40%, a throat symptom was also the most common, followed by symptoms of the teeth or gums (Figure 1). Symptoms such as inflammation, skin infection, or diarrhea were reported only in countries with lower response rates. Self-medication for pyelonephritis or pyelitis was reported only in Lithuania; diarrhea was reported in Lithuania (10 patients; 9 used chloramphenicol), Austria (1 patient), and United Kingdom (1 patient).

For intended self-medication as with actual self-medication, a sore throat was the most common symptom, followed by urinary tract infection and toothache (Figure 2). When including only those countries that had response rates >40%, sore throat and urinary tract infection were also the most common symptoms (Figure 2).

In eastern countries, the major source of self-medication was the pharmacy without prescription (309 courses, 68%), followed by leftover medications (120 courses, 26%). By contrast, in southern, northern, and western countries use of leftover medication was more prevalent (46 courses [51%] in southern countries and 35 courses [44%] in northern and western countries), followed by medications obtained directly from the pharmacy (41 courses [46%] in southern countries and 15 courses [19%] in northern and western countries). Among other sources of self-medication were drugs obtained from relatives or friends (52 courses, 8%, in all countries), drugs that were

[2]The adjusted estimates for each country are available from the corresponding author on request.

Table 1. General characteristics of respondents in each European country

Country	Response rate (%)	No. respondents	Mean age (y) ± SD	Female (%)	Low education level* (%)	Presence of chronic disease† (%)	Urban location (%)
Northern and western							
The Netherlands	55	1,634	48 ± 17	58	30	15	48
Sweden	69	704	54 ± 19	53	53	14	49
Denmark	63	1,881	48 ± 17	56	60	14	49
Austria	28	442	49 ± 16	50	64	15	53
Belgium	54	1,734	45 ± 16	55	32	13	50
Luxemberg	50	675	46 ± 18	51	49	15	45
United Kingdom‡	23	675	50 ± 10	58	40	15	58
Ireland	26	793	48 ± 16	59	53	17	47
Southern							
Israel	18	467	50 ± 17	61	19	22	36
Malta	54	541	46 ± 16	55	60	21	47
Italy	21	213	45 ± 18	61	37	27	51
Spain	20	204	47 ± 14	47	31	18	57
Eastern							
Czech Republic	59	1,169	54 ± 15	36	45	27	48
Slovenia	38	1,143	48 ± 17	58	70	20	47
Croatia	31	615	53 ± 16	55	10	26	58
Poland	32	935	45 ± 18	60	42	23	52
Slovakia	55	546	41 ± 16	54	27	23	54
Romania	43	430	50 ± 18	49	43	27	43
Lithuania	25	747	59 ± 18	35	32	39	54

*Low education level was defined as incomplete primary education, completed primary education, and lower vocational or general education.
†Including any of the following diseases: asthma, chronic bronchitis, emphysema, human immunodeficiency virus infection, cystic fibrosis, diabetes, endocarditis, tuberculosis, prostatitis, chronic urinary tract infection, chronic osteomyelitis, peptic ulcer disease, chronic pyelonephritis or cancer.
‡Reminders were not sent to nonrespondents.

stored after being obtained abroad (10 courses, 2%, in all countries), and drugs obtained over the Internet (3 courses, all in Lithuania).

Effects of Individual Characteristics

The effects of demographic characteristics and chronic disease on actual self-medication, intended self-medication, and storage of antimicrobial drugs are shown in Table 3. Sex and location (urban or rural) had no significant relevance in any of the 3 models. Respondents from southern and eastern countries were more likely to self-medicate (adjusted odds ratio [OR] 6.8, 95% CI 4.8–9.7, and 7.5, 5.7–10.0, respectively) than respondents from northern and western countries. Younger age, higher educational level, and presence of a chronic disease were all significantly associated with self-medication. Similar results were obtained for the relationship between demographic characteristics and storage of antimicrobial drugs, by using the conservative estimate of storage. Younger age, higher educational level, and presence of a chronic disease were also significant predictors of intended self-medication. Presence of a chronic disease increased the risk of intended self-medication, but this effect diminished with increasing age. We repeated all analyses including only those countries that had response rates >40% and obtained similar results. We also repeated these analyses separately for early and late respondents and obtained similar results.[3]

Relation between Intended Self-medication, Storage, and Actual Self-medication

Intended self-medication and storage are both predictors of actual self-medication. A significant relationship was found between intended self-medication and storage. Intended self-medication was a strong predictor for actual self-medication for both respondents who stored drugs (OR 20.9, 95% CI 15.5–28.2) and those who did not (OR 17.8, 95% CI 14.0–22.7). However, for those who did not intend to self-medicate, storage also predicted higher actual self-medication (OR 3.5, 95% CI 2.2–5.6). When the analyses were repeated, including only those countries that had response rates >40%, similar results were obtained.[3]

Conclusions

Self-medication with antimicrobial drugs occurred in all countries that participated in this survey. We included the data from both countries that had high and low response rates. In most of the countries with low response rates (except Spain), no other information is available about self-medication, an often overlooked issue. The second reason for including these countries was that low response was not a problem of this study only, but a general problem of surveys in these countries (*29,30*). This finding implies that if we want to include information

[3]Results are available from the corresponding author on request.

Table 2. Actual use of systemic antimicrobial drugs in the last 12 months and at-risk self-medication in 19 European countries

	Rate per 1,000 respondents (95% confidence interval)				
Country (region in country)	Actual self-medication	Prescribed use	Intended self-medication	Storage* (conservative estimate)	Storage† (maximum estimate)
Countries with response rate ≥40%					
Northern and western					
The Netherlands (Twente)	1 (0.2–4)	152 (134–170)	85 (71–101)	10 (6–17)	36 (28–46)
Sweden (Vastmanland)	4 (0.9–12)	135 (109–161)	118 (94–143)	14 (7–26)	43 (29–60)
Denmark (Funen, Aarhus, Copenhagen‡)	7 (4–12)	172 (154–189)	132 (116–147)	42 (33–52)	84 (72–97)
Luxemburg (whole country)	9 (3–19)	288 (252–324)	83 (62–107)	90 (69–114)	132 (106–158)
Belgium (East Flanders, Flemish Brabant)	9 (5–15)	222 (201–242)	80 (67–95)	71 (59–84)	123 (107–138)
Southern					
Malta (whole country)	56 (38–79)	422 (380–465)	228 (192–264)	156 (125–186)	269 (232–306)
Eastern					
Czech Republic (Hradec Krlov)	7 (3–13)	253 (228–279)	179 (156–201)	45 (33–58)	64 (51–80)
Slovakia (Middle Slovakia region)	42 (27–63)	569 (527–612)	324 (284–365)	192 (159–225)	302 (263–340)
Romania (Dolj)	198 (160–235)	307 (263–351)	431 (383–478)	200 (162–238)	321 (277–365)
Countries with response rate <40%§					
Northern and western					52 (33–77)
Austria (Upper Austria)	9 (2–23)	159 (124–195)	73 (49–103)	34 (19–55)	52 (33–77)
United Kingdom (Nottinghamshire)	12 (5–23)	221 (189–254)	166 (137–195)	33 (21–49)	74 (56–97)
Ireland (Cork)	14 (7–25)	353 (320–386)	150 (125–176)	29 (19–43)	100 (80–123)
Southern					
Israel (Northern Israel)	15 (6–31)	330 (287–374)	187 (150–223)	120 (91–149)	236 (197–274)
Italy (Abruzzo)	62 (33–103)	512 (444–580)	243 (185–301)	379 (314–445)	569 (502–636)
Spain (autonomous community of Madrid)	152 (103–201)	315 (251–379)	314 (249–380)	260 (200–320)	500 (431–569)
Eastern					
Slovenia (Ljubljana region)	17 (10–26)	293 (266–320)	280 (253–307)	119 (100–137)	183 (160–205)
Croatia (Zagreb county)	31 (19–48)	439 (399–478)	205 (172–237)	130 (103–156)	212 (179–244)
Poland (Pomorskie)	33 (23–47)	199 (172–225)	115 (94–136)	69 (53–87)	137 (115–160)
Lithuania (Klaipeda, Rietavas)	210 (181–239)	275 (243–308)	449 (412–486)	177 (149–204)	333 (299–367)

*Included only those respondents who stored antimicrobial drugs and had not taken the same antimicrobial drugs for a prescribed course in the previous 12 months.
† Including all respondents who stored antimicrobial drugs.
‡ Although Copenhagen has population >750,000, both self-medication and prescribed use of antimicrobial drugs were not significantly different between the sample of Copenhagen and sample of the other 2 Danish counties (χ^2 tests).
§The rates for these countries should be interpreted as first rough estimates.

about these countries, the results may be biased. In addition, debate is growing that low response is less problematic in affecting survey estimates than previously assumed (*31*). Nevertheless, the prevalence rates of self-medication in countries with low response rates should be considered as a rough estimate and interpreted as an indication that the problem exists.

Antimicrobial drug self-medication prevalence varies widely among different European regions, with the highest rates in eastern and southern countries, and the lowest in northern and western. Besides actual self-medication, intended self-medication is clearly relevant: it is a strong predictor of actual self-medication. Intended self-medication has a much higher prevalence than actual self-medication, indicating that the population at-risk is much larger than those who have actually self-medicated in the previous 12 months. Another risk factor for actual self-medication is the availability of drugs at home; opportunity encourages use. Our findings contribute to the growing evidence that estimates of antimicrobial drug use that are based on prescription data only are likely to underestimate actual consumption in both Europe and the United States (*11,32*). Our European estimates are low in comparison with those from a recent study in the US Hispanic community that showed that ≈20% of the respondents acknowledged getting drugs without a prescription in the United States (*32*). The only comparable high rates were found in Spain, Romania, and Lithuania, where they ranged from 9% to 18%. However, these figures should be compared with caution because our estimates refer to acquiring drugs without prescription in the last 12 months and the United States study refers to ever acquiring them.

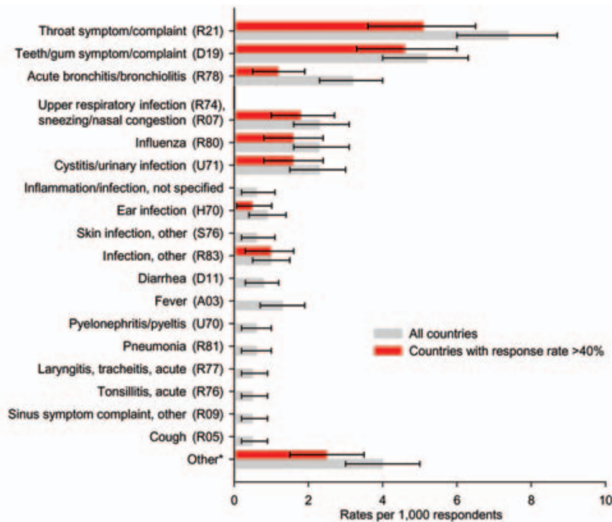

Figure 1. Prevalence of actual self-medication by symptoms or diseases classified by International Classification of Primary Care codes (rates per 1,000 respondents and 95% confidence interval). *Symptoms or diseases with rates <1 per 1,000 respondents, including eye infection, pain, prostatitis, urogenital infection, headache, and "bad health."

We found that many persons used antimicrobial drug leftovers from previous prescriptions, as was the case in reports from the United States (*8–10,12*). Drugs could be left over because extra tablets were dispensed (in many countries pharmacies dispense drugs per package, not exact number of tablets) or because of patient noncompliance. Noncompliance may result in 2 inappropriate courses if the patient does not take the amount of medication prescribed and self-medicates later. Earlier findings indicated lower compliance in Italy and Spain than in Belgium, France, and the United Kingdom (*33*). In Italy, 41% of the interviewees who had taken drugs in the previous 12 months saved part of the course for future use, whereas only 4% of British interviewees reported this behavior (*33*).

In general, respondents' self-diagnosed disorders were self-limiting and antimicrobial drugs would not have been indicated. In contrast to studies in developing countries, this study identified few cases of self-medication for sexually transmitted diseases (*34,35*). Only 2 respondents in Lithuania reported self-medication for "gynecological infection" that might have been a sexually transmitted infection.

In this survey, persons who were more prone to self-medicate with antimicrobial drugs were younger persons, more educated, and had chronic diseases. This finding corresponds to those of studies conducted in the United States and Greece, which also found that higher educational status is associated with misuse of drugs (*8,17*). This relationship cannot be directly attributed to educational status. The interpretation of symptoms is also relevant. Previously, a study in the United States showed that persons with a higher education level tended to believe that antimicrobial drugs were less effective for upper respiratory infections with clear discharge but more effective with discolored discharge (*36*).

Antimicrobial drug self-medication is a cause for concern because it may contribute to the spread of antimicrobial drug resistance. Self-treatment with a drug that is ineffective against the causative organism or with an inappropriate dosage may increase the risk of selection of resistant organisms that are difficult to eradicate. These resistant organisms may then be transferred into the community. Our findings illustrate that adverse effects are aggravated by self-medication when unnecessary drugs, such as chloramphenicol, tetracycline, and aminoglycosides, are taken. Other problems related to self-medication include drug interactions, masked diagnoses, and superinfection.

Our results are comparable to those of other studies such as the Eurobarometer study (*14,19,27*). A study on antimicrobial drug storage among Spanish households showed that 42% of Spanish households had drugs at home, including those currently used (*14*). This finding is comparable to the prevalence of drugs stored (50%) in our study. In Malta, a higher prevalence (19%) of self-medication was found (*19*) than in our study, perhaps because the study included self-medication in the previous 2 years, while our study included the previous 12 months. Furthermore, the pattern of the prescribed use of drugs in different regions of Europe in our study is similar to that found in the study by Goossens et al., which was based on information from national databases (*4*).

A strength of our study is that we used the same methods and comparable samples in all countries, which facilitated an overview of the European situation. The low

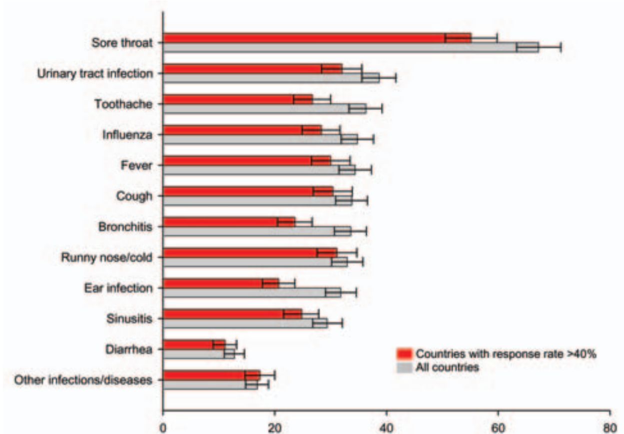

Figure 2. Prevalence of intended self-medication per predefined symptom (rates per 1,000 respondents and 95% confidence interval).

Table 3. Effects of individual characteristics on actual and at-risk antimicrobial drug self-medication*

Characteristics	Adjusted odds ratio (95% confidence interval)		
	Actual self-medication	Storage (conservative estimate)	Intended self-medication
Age	0.985 (0.979–0.992)	0.977 (0.973–0.982)	0.984 (0.980–0.987)
Region in Europe†			
Northern and western	1 (reference)	1	1
Southern	6.776 (4.752–9.662)	5.101 (4.240–6.137)	2.233 (1.909–2.613)
Eastern	7.529 (5.676–9.985)	3.311 (2.868–3.822)	2.851 (2.577–3.154)
Education level			
Low‡	1 (reference)	1	1
High	1.357 (1.095–1.680)	1.690 (1.470–1.943)	1.233 (1.116–1.361)
Chronic disease§			
No	1 (reference)	1	1
Any	1.888 (1.497–2.383)	1.225 (1.038–1.446)	2.320(1.594–3.378)
Age × any chronic disease			0.989 (0.982–0.996)
Exponential (constant)	0.012	0.083	0.219

*At-risk self-medication included intended self-medication or storage of drugs at home.
†Northern and western includes Sweden, Denmark, the Netherlands, Austria, Belgium, Luxemburg, United Kingdom, Ireland; southern includes Israel, Malta, Italy, and Spain; eastern includes Czech Republic, Slovenia, Croatia, Poland, Slovakia, Romania, and Lithuania.
‡Low education level was defined as incomplete primary education, completed primary education, and lower vocational or general education.
§Including any of the following diseases: asthma, chronic bronchitis, emphysema, HIV infection, cystic fibrosis, diabetes, endocarditis, tuberculosis, prostatitis, chronic urinary tract infection, chronic osteomyelitis, peptic ulcer disease, chronic pyelonephritis, or cancer.

response rate in some countries is a limitation of our study, however. Although we calculated the prevalence rates adjusted for nonresponse, they are based on the assumption that respondents who replied after the reminder most resemble nonrespondents.

As with all self-reported data, results of this survey have the potential for recall bias, underreporting, or overreporting. We attached the list of the most commonly used antimicrobial drugs in each country to the questionnaires to reduce recall problems. To discourage underreporting of self-medication, the questions about drug use were formulated in a neutral way in which the source of the drug could be chosen from 6 predefined sources or "other source."

Substantial variation in the prevalence rates of antimicrobial drug self-medication among the European regions suggests that cultural (*37*) and socioeconomic factors play a role, as do disparities in health care systems such as reimbursement policies, access to health care, and drug dispensing policies. Another factor is the acquisition of antimicrobial drugs from pharmacies without prescription, which occurred most frequently in eastern European countries. Although over-the-counter sale of antimicrobial drugs is illegal in all participating countries, there is clearly a need to enforce the law in some countries.

Antimicrobial drug self-medication is a cause for concern in Europe. Even the lowest prevalence, 1 person per 1,000 respondents, implies that 10,000 persons in a population of 10,000,000 are self-medicating annually. Our study indicates a high prevalence of self-medication in countries that reported high resistance levels (southern and eastern countries). Even in the countries with low actual self-medication, substantial intended self-medication and drug storage occurs. Efforts to reduce inappropriate use of antimicrobial drugs should include the issue of self-medication and should involve prescribers, pharmacists, and the general public. The number of tablets dispensed in pharmacies should be limited, and patients should be instructed to discard their leftover drugs. Large-scale public campaigns, such as those recently launched in the United States, Canada, Belgium, and Australia (*38*), should include detailed instructions and emphasize the potential risks of using antimicrobial drugs without medical guidance.

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Pneumonic Plague Cluster, Uganda, 2004

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The public and clinicians have long-held beliefs that pneumonic plague is highly contagious; inappropriate alarm and panic have occurred during outbreaks. We investigated communicability in a naturally occurring pneumonic plague cluster. We defined a probable pneumonic plague case as an acute-onset respiratory illness with bloody sputum during December 2004 in Kango Subcounty, Uganda. A definite case was a probable case with laboratory evidence of *Yersinia pestis* infection. The cluster (1 definite and 3 probable cases) consisted of 2 concurrent index patient–caregiver pairs. Direct fluorescent antibody microscopy and polymerase chain reaction testing on the only surviving patient's sputum verified plague infection. Both index patients transmitted pneumonic plague to only 1 caregiver each, despite 23 additional untreated close contacts (attack rate 8%). Person-to-person transmission was compatible with transmission by respiratory droplets, rather than aerosols, and only a few close contacts, all within droplet range, became ill.

Naturally occurring plague occurs most frequently in bubonic or septicemic forms and is usually acquired through the bite of an infected rodent flea. Bubonic and septicemic plague are not transmissible from person to person, but if left untreated, plague bacteria can spread hematogenously to the lungs, resulting in secondary pneumonic plague. Pneumonic plague is contagious through infectious respiratory secretions, potentially resulting in direct airway infection (primary pneumonic plague) among close contacts (*1*,*2*).

Pneumonic plague epidemics in China early in the 20th century killed tens of thousands of persons (*3*). Plague is now rare in developed countries. However, the possibility of an intentional aerosol release of plague bacteria causing numerous contagious primary pneumonic plague cases has been a top concern of bioterrorism specialists (*1*). Consequently, *Yersinia pestis* release scenarios have been used in large-scale bioterrorism preparedness drills (*4,5*). The possibility of pneumonic plague importation's causing an outbreak in a nonendemic region is also a concern (*6*).

In-country panic and international alarm followed the 1994 report of pneumonic plague in India (*7*). Physicians reportedly fled Surat, the affected city, stating that there was "nothing to be done," and tetracycline was hoarded in areas distant from the reported outbreak (*7*). Some commercial airline flights (*8*) and exports (*7*) from India were cancelled. English physicians contested their public health officials' description of plague's low communicability based on their clinical training and infectious disease textbooks (*9*). Commercial repercussions for India have been estimated at US $3–$4 billion (*7*). Similarly and more recently, thousands fled a suspected pneumonic plague outbreak in the Congo during 2005 (*10*).

The public and clinicians have long-held beliefs that pneumonic plague is highly communicable (*9,11–13*). The current Infectious Diseases Society of America (IDSA) summary on *Y. pestis* as a bioterrorism agent notes secondary transmission risk is not well-quantified (*14*). Because of its rarity, recent published observations on its contagiousness are scarce, and few clinicians have first-hand knowledge of the disease.

We describe pneumonic plague's communicability and clinical course in a recently investigated cluster. On December 26, 2004, a Ugandan police officer telephoned a local physician (author G.A.) about a cluster of deaths in that country's West Nile region. The physician initiated an investigation that day and was joined the next day by US Centers for Disease Control and Prevention (CDC) staff who were in the area for a plague treatment trial.

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Methods

The surviving patient, caregivers and healthcare providers of the ill, and other close contacts of the deceased were interviewed to understand how the outbreak was propagated and to identify close contacts needing prophylaxis. The surviving patient's clinicians (G.A. and Z.A.) provided clinical information. Because CDC and the Ugandan Ministry of Health were conducting a plague treatment trial in the area, prospective enhanced surveillance for plague was already ongoing in the West Nile region, involving at least weekly local health center visits. For this enhanced surveillance, a probable bubonic plague case was defined as an illness with fever and tender lymphadenopathy without another cause for lymphadenopathy (e.g., cellulitis or streptococcal pharyngitis). For this cluster investigation, we conducted additional retrospective pneumonic plague surveillance by interviewing private drug shop owners, business owners, traditional healers, and other area residents. We defined a probable pneumonic plague case as respiratory illness of acute onset with cough producing grossly bloody sputum during December 2004 in Kango Subcounty, Nebbi District, Uganda. For this investigation, we defined a definite pneumonic plague case as a probable case with laboratory evidence of plague infection.

Laboratory Methods

The surviving patient's sputum and serum samples were tested for direct and indirect evidence of plague infection. Sputum was placed on blood agar plates to recover live organisms, tested by polymerase chain reaction (PCR) for evidence of *Y. pestis* DNA, reacted with fluorescent-labeled antibody specific for *Y. pestis* and analyzed by fluorescent microscopy, and assessed for *Y. pestis* antigen by using 2 handheld immunochromatographic assays (i.e., dipsticks) (TetraCore, Inc., Gaithersburg, MD, USA, and New Horizons, Columbia, MD, USA). Serum samples collected during the acute phase of the disease were tested for antibody to F1 antigen of *Y. pestis* (*15*).

Extraction of DNA and PCR

The genes *caf1*, *repA1*, and *pla* were analyzed by PCR. CDC has used these primers for many years for recognition of *Y. pestis* DNA. Primer sequences were *caf1*-f 5′-ATACTGCAGATGAAAAAAATCAGTTCC-3′, *caf1-r* 5′-ATAAAGCTTTTATTG GTTAGATACGGT-3′; *repA1-f* 5′-AGGCCCTGTTCACACATC-3′, *repA1-r* 5′-CCGGG TGTA GTTATTGTTCC-3′; and *pla-f* 5′-ATCTTACTTTC-CGTGAGAA-3′, *pla-r* 5′-CTTGGATGTTGA GCTTCC-TA-3′. Basic local alignment and sequencing tool analysis against all known sequences in GenBank demonstrated no significant homologies outside *Y. pestis* for *caf1* and *pla* primers. The *repA1* primer set also has 100% homology to *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. DNA was extracted from 200 µL of sputum by using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) and manufacturer's protocol. A total of 5 µL extracted DNA from the sputum or a positive control (*Y. pestis* strain CO 92) or negative control (water) was added to each reaction. PCR conditions were as previously described (*16*). Expected amplicon sizes were 531 bp (*caf1*), 833 bp (*repA1*), and 480 bp (*pla*). PCR was carried out by using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ, USA).

Direct Fluorescent-Antibody Test

Sputum was vigorously vortexed to disrupt the semisolid mass, then centrifuged at 8,000 × *g* for 5 min to pellet the solid material. The pellet was washed once in phosphate-buffered saline (PBS) and resuspended in 75 µL of PBS. Approximately 5 µL of concentrated sputum was used for direct fluorescent-antibody (DFA) microscopy as previously described (*17*) and visualized at 400× magnification.

Results

Cluster Description

We identified 1 definite and 3 probable pneumonic plague cases, comprising 2 concurrent index patient–caregiver pairs. We refer to the pairs as A and B, with numbers 1 and 2 designating index and caregiver cases within each pair, respectively. Index patient B1 became ill 1 day before cough onset in index patient A1. Despite extensive investigation, we identified no social links between these 2 index patients and no evidence of contact in the week before patient A1's illness onset. We identified no other illnesses clinically compatible with pneumonic plague occurring in December 2004 in Kango Subcounty by active surveillance. All case-patients' disease symptoms are shown by onset day in the Table. Overall, compared with index patients, caregivers' illnesses progressed more rapidly, including quicker bloody sputum onset (mean 1 vs. 6 days).

Index patient A1 was a 22-year-old woman, and her primary caregiver, patient A2, was her 40-year-old mother. According to family members, patient A1's illness began with several days of headache, fever, and chills. Lymphadenopathy was first observed on day 3. Coughing, first noted on day 5, became productive a day later and bloody sputum was noted on day 7. On day 6, she was seen by a drug shop owner (a government-trained nursing assistant) and treated for malaria with 3 days of chloroquine. On day 9, she was coughing frank blood and died later that

Table. National history and timing of symptom onset for index patients (secondary pneumonic plague) versus caregiver-patients (primary pneumonic plague), Uganda, December 2004

		Symptom onset by day of illness*																	
		1		2		3		4		5		6		7		8		9	
Symptoms†	Patient‡	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
Fever/chills	A1	X																	
	B1	X																	
	A2	O																	
	B2	O																	
Headache	A1	X																	
	B1	X																	
	A2	O																	
	B2	O																	
Lymphadenopathy	A1					X													
	B1	Unknown																	
	A2	None																	
	B2	None																	
Weakness	A1							X											
	B1				X														
	A2	O																	
	B2	O																	
Cough	A1									X									
	B1								X										
	A2	O																	
	B2		O																
Chest pain	A1									X									
	B1												X§						
	A2	O																	
	B2	O																	
Productive cough	A1													X					
	B1									X									
	A2	O																	
	B2		O																
Bloody sputum	A1															X			
	B1										X								
	A2				O														
	B2		O																
Shortness of breath	A1																	X	
	B1											X							
	A2			O															
	B2			O															
Nonambulatory	A1																	X	
	B1													X					
	A2					O													
	B2	Never																	
Death/effective treatment	A1																		X¶
	B1														X¶				
	A2						O¶												
	B2				O#														

*X, secondary pneumonic plague index patient; O, primary pneumonic plague caregiver–patient. AM, symptom first appeared between midnight and noon; PM, symptom first appeared between noon and midnight.
†Additional symptoms: caregiver A2 had a sore throat and ulcerative pharyngitis. Caregiver B2 had a burning back pain that started at the time of illness onset. Index patients A1 and B1 both had vomiting, and B1 also had diarrhea.
‡Cases are listed by time of onset: 12/9 (A1), 12/13 (B1), 12/23 (A2), 12/25 (B2). Caregiver A2 reported her husband was ill about midday on 12/23, but symptoms might have started earlier. Caregiver A2 was index patient A1's mother and her primary caregiver during her illness. Caregiver B2 was index patient B1's sister and his primary caregiver during his illness.
§Patient B1 first reported to family members severe chest pain on day 6 of illness but might have had mild chest pain earlier.
¶Death.
#Treatment and survival.

night. Patient A1's primary caregiver, patient A2, became ill 5 days after her daughter's death. On the first day of patient A2's illness, she reported headache, fever, chills, weakness, chest pain, and a productive cough. The same private drug shop owner examined her and reported ulcerative pharyngitis, a sign associated with inhalational exposure to *Y. pestis* (*12*), but not lymphadenopathy. The patient was treated with intramuscular penicillin (6 treatments over 36 h) for presumptive severe pneumonia. The next day grossly bloody sputum developed, and she died on illness day 3.

Index patient B1 was a 25-year-old man, and his primary caregiver, patient B2, was his 30-year-old sister. Index patient B1's illness began with headache, fever, and chills. His family sought care for him at a private drug shop and transported him to 3 government health centers. Lymphadenopathy was not reported, although his clinicians did not specifically examine him for it. He received antimalaria treatment and intramuscular penicillin for presumptive severe pneumonia. His cough became productive with bloody sputum on day 5 of illness, and he died on day 6. Patient B1's primary caregiver, B2, became ill 5 days after patient B1's death.

Surviving caregiver B2 was identified the day the outbreak was reported, a day after her illness onset. She was markedly dyspneic, ill-appearing, with an elevated oral temperature and respiratory rate (39.3°C and 56 breaths/min, respectively). She required assistance to walk. She had no palpable lymph nodes. A pulmonary examination showed marked chest indrawing and bilateral coarse crepitations. She was first treated 29 h after illness onset at the local health center, where she received chloramphenicol, 2 g intravenously as a single bolus, and doxycycline, 100 mg orally.

On her arrival at Nyapea Hospital, a grossly bloody sputum sample was obtained (Figure 1A). Because hospital staff were unaware of her previous treatment, she was retreated with chloramphenicol, 1 g intravenously, given 1 h and 45 min after her initial dose. She continued treatment with chloramphenicol, 1 g intravenously every 6 h for 48 h, then 750 mg every 6 h (10 days of intravenous treatment). She was discharged at day 10 and received oral chloramphenicol, 750 mg every 6 h for 8 additional days. On discharge, she was able to walk 1 mile to her home from the nearest road but with difficulty and shortness of breath. Three weeks after being discharged, she reported having returned to all usual activities including subsistence farming.

A series of 3 frontal chest radiographs taken on days 2, 3, and 18 of illness demonstrated bilateral airspace disease, predominantly in lower lung zones, with bilateral (right > left) pleural effusions without evidence of hilar or mediastinal lymphadenopathy (Figure 2). Findings were consistent with multilobar pneumonia with progressive diminution in airspace disease and pleural effusions over time.

Presence of *Y. pestis* in the surviving patient's sputum was verified by positive PCR results for genes on all 3 of the *Y. pestis* plasmids (Figure 1B), and DFA showing classic fluorescent staining halos of bacteria with *Y. pestis*-specific antibody (Figure 1C). Two handheld assays also detected *Y. pestis* antigen in the sputum. The sputum, which was obtained 1.5 h after her first antimicrobial drug dose, stored overnight without refrigeration, and transported the next day to the central laboratory (6 h in transport), did not yield *Y. pestis* on bacterial culture. A complete blood count at illness day 20 was within normal limits. Antibody to *Y. pestis* was not detected in serum from acute-phase blood samples. The patient declined to provide a sample for convalescent-phase serologic testing at a follow-up visit 3 weeks after discharge. The other 3 case-patients were already buried when the outbreak was reported; therefore, autopsies and laboratory verification of their plague diagnoses were not attempted.

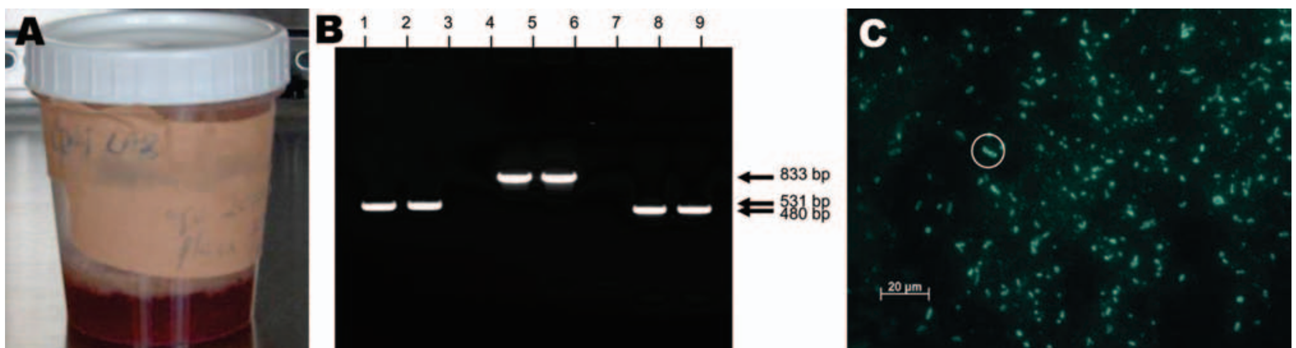

Figure 1. A) Grossly bloody sputum sample obtained from the surviving patient (caregiver B2) 30 h after onset of primary pneumonic plague. B) Polymerase chain reaction results of sputum sample from caregiver B2. Lanes 1–3, *caf1*; lanes 4–6, *repA1*; lanes 7–9, *pla*. Lanes 1, 4, and 7 are positive controls; lanes 2, 5, and 8 are patient samples; lanes 3, 6, and 9 are negative controls. C) Anti-F1 direct fluorescent antibody staining of sputum sample from caregiver B2. Numerous bacteria with classic halo structures are characteristic of *Yersinia pestis*. The circled bacterium classically depicts this halo.

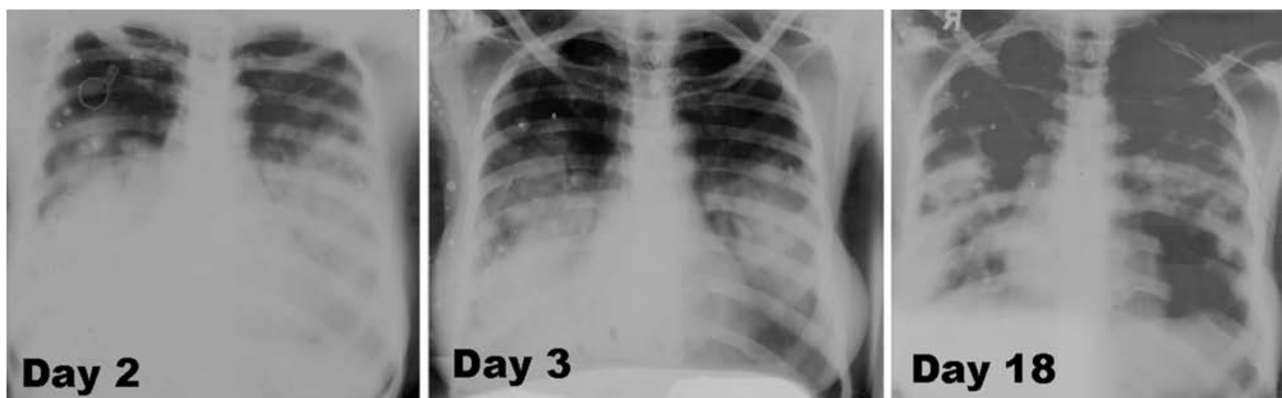

Figure 2. Serial frontal chest radiographs from surviving caregiver B2 with primary pneumonic plague obtained on illness days 2, 3, and 18, showing bilateral lower lung zone predominant airspace disease associated with right>left pleural effusions. The radiographs have artifacts related to hand-dipping of films, which account for multiple densities that move between images and areas of apparent lucency.

Contact Tracing and Prophylaxis

Close contacts of index patients A1 and B1 are described in the Appendix Table (available from http://www.cdc.gov/ncidod/EID/vol12no03/05-1051_app.htm). These contacts were not given antimicrobial drug prophylaxis because >1 week had passed since the index patients' deaths when the outbreak was reported. Twenty-five persons had direct contact with either patient (i.e., touched) after onset of cough productive of bloody sputum and before death, but only the 2 primary caregivers became ill (attack rate 2/25, 8%).

Examples of these index patients' close contacts include the following. Patient A1 slept in the same bed as her husband and 1.5-year-old daughter in a 1.8 × 3.1 × 2.0m bedroom the night before her death. The night before index patient B1's death, he slept in the same bed as his 6-year-old daughter until the early morning, when his wife noted he was very ill and coughing bloody sputum. His daughter then moved to a straw mat on the floor of the 4 × 4 × 1.6 m windowless 1-room house with her mother and 3 siblings, who had been sleeping there. Their heads were ≈1.8 m from their father's.

On index patient B1's last day, he was placed in a chair strapped on the back of a bicycle and transported 18 km to obtain medical care at several clinics. His 3 brothers who held him upright during this trip remained well without prophylaxis. In addition, ≈200 persons attended the 2 index patients' funerals; ≈75 persons touched the blanket that wrapped index patient B1's body, the same blanket that was used during the patient's final days of illness. No contacts used masks, gloves, or any other form of respiratory protection.

All identified close contacts of caregivers A2 and B2 received chemoprophylaxis (3 days of cotrimoxazole, 960 mg orally, twice a day), including 14 members of caregiver A2's family compound, 8 members of caregiver B2's family compound, and 4 healthcare providers who rode without masks in the ambulance with caregiver B2. Prophylaxis was initiated 4 days after caregiver A2's death, 2 days after caregiver B2's treatment initiation, and on the day of the ambulance ride, respectively. Additionally, local health authorities gave prophylaxis to 200 attendees of caregiver A2's funeral on the day of the funeral, which took place the morning after A2's death, the same day the outbreak was reported.

Community Surveillance

No additional pneumonic plague cases were identified during December and in the weeks after the outbreak report. However, through active surveillance we identified 3 probable bubonic plague patients who came to the subcounty's local health center in the first half of January, an increase from a baseline of 0 cases per month in the preceding 3 months. In addition, during the investigation in late December and early January, several villages in the subcounty reported rat deaths, and both index patients' families reported finding dead rats in their family compounds before the index patients' illness onset.

Discussion

We report 4 pneumonic plague cases involving 2 instances of person-to-person transmission. Even without appropriate treatment, the 2 index patients survived ≥1 week. The index patients transmitted pneumonic plague, likely in their final hours of life, to only their primary caregivers, despite numerous other close contacts. This transmission pattern is compatible with respiratory droplet transmission rather than transmission by aerosols (droplet nuclei). Furthermore, only a few close contacts, who were all within droplet range, became ill. Primary pneumonic plague developed in the primary caregivers, who displayed a more fulminant clinical course. However, 1 survived without residual functional limitation after chloramphenicol treatment initiated 29 h after illness

onset, which is later than commonly thought useful (*11,12,14*).

We identified 23 close contacts of the 2 index patients who remained well without antimicrobial drug prophylaxis or other form of protection, including 3 family members who slept in the same bed and many persons who slept with their heads at a distance <2 m from the coughing plague patient. Some published literature describes pneumonic plague as highly contagious (*9,12*) through aerosols (droplet nuclei) (*13*). However, other researchers have reported that transmission requires prolonged close contact at the end stage of illness (*2,18,19*), which is consistent with respiratory droplet transmission (*1,20*). This investigation supports the latter view. Furthermore, droplet range is usually ≤3 feet (*21*), and all identified close contacts were well within that proximity to an index patient, but few (8%) became ill. Transmission likely occurred on the index patients' final day of life, given the 5-day interval until caregiver symptom onset after the index patients' death (incubation period usually 2–4 days, range 1–6) (*2,19*). Consistent with our findings, Gani and Leach's review of pneumonic plague outbreaks reported an average 1.3 pneumonic plague transmissions to other persons per pneumonic plague case (*22*). An investigation of a larger outbreak in Madagascar that used serologic testing to confirm plague infections also reported an attack rate among close contacts similar to ours (8.4%), although a definition of close contacts was not reported (*23*).

Our patients' clinical course provides clues to why pneumonic plague patients usually infect few persons and why, for example, an air travel–associated outbreak would be unlikely. Our case-patients were visibly short of breath, coughing grossly bloody sputum, and barely ambulatory before transmitting the disease. Thus, when patients are substantially contagious, they are unlikely to be traveling by air and, if so, would appear ill enough to alarm nearby passengers. In most settings, persons this ill are at home or in the hospital. Recent reviews support this observation because most reported pneumonic plague transmissions involve family, friends, or medical professionals caring for ill persons at home or in the hospital (*2,22*).

A current IDSA summary on *Y. pestis* as a bioterrorism agent notes, "in the absence of early therapy (i.e., within the first 24 h), death occurs from overwhelming sepsis" (*14*). This follows Butler's widely cited reviews, which state that pneumonic plague is "invariably fatal" if treated >20–24 h after illness onset (*11,12*) and cite the 1956 report of McCrumb et al. (*24*). More recent reviews (*25*) and other literature (*26,27*) indicate that survival is possible even when treatment is initiated after 24 h, consistent with caregiver B2's survival. This caregiver received chloramphenicol, the only parenteral drug designated as a national standard plague treatment in Uganda (*28*), 29 h after illness onset, and survived without supportive care (i.e., mechanical ventilation or oxygen therapy). In the United States, chloramphenicol is a second- or third-line plague treatment (*1,29*) because no randomized clinical trials have been conducted to document its comparability with accepted treatments and because it has potential hematologic side effects (*1*). Although caregiver B2 received supratherapeutic doses because of a communication error, experienced Ugandan clinicians report success treating plague, including pneumonic plague, with chloramphenicol, 500 mg intravenously every 6 h (*30*).

Clinical diagnosis of pneumonic plague is challenging, particularly without lymphadenopathy. Even in this plague-endemic area these cases were not suspected to be plague until an investigation was initiated after the third death. All 4 cases had 1 classic pneumonic plague feature: productive cough progressing to grossly bloody sputum (*2,18,19*). In plague-endemic regions, respiratory illnesses <1 week in duration with bloody or blood-tinged sputum should prompt consideration of a pneumonic plague diagnosis and empiric antimicrobial drug treatment for plague. Routine chest radiographs cannot be expected to establish a pneumonic plague diagnosis. The chest radiographs of surviving caregiver B2 were consistent with but not uniquely diagnostic for primary pneumonic plague. As with the few other published radiographs of primary pneumonic plague patients (*1,24,27,31–33*), these radiographic findings alone would not prompt clinicians to consider pneumonic plague without a preexisting clinical suspicion.

In our cluster, primary pneumonic plague (direct airway infection) progressed rapidly to life-threatening illness. In contrast, respiratory symptoms developed later in illness for the apparent secondary pneumonic plague patients, consistent with hematogenous spread from an alternate site of infection initiation, and their symptoms progressed more slowly. The more fulminant clinical course of primary pneumonic plague could help differentiate primary versus secondary pneumonic plague in naturally occurring outbreaks and pneumonic plague suspected of being caused by an intentional bacterial release because aerosol exposure would result in primary pneumonic plague. Time course of clinical progression can be established retrospectively from history alone, in contrast to lymphadenopathy (a bubo), which can also help differentiate primary and secondary pneumonic plague but requires a thorough physical examination. A bubo indicates that pneumonic plague is most likely secondary to a primary bubonic plague infection (*14*).

Upon hearing of 4 cases in close proximity, our initial assumption was these cases were linked. However, closer investigation demonstrated that the second patient became ill before the first patient developed cough, and these 2 patients had no apparent contact during the week before

the second patient's illness onset. They lived within 2 km of each other but were from different villages and tribal backgrounds. This coincidence indicates the importance of detailed contact investigations before pneumonic plague cases are declared linked in areas with ongoing epizootics. The cluster is likely explained by the annual epizootic reaching the area and difficulty diagnosing pneumonic plague. Because the 2 index patients lived near each other, they were likely both exposed to the same epizootic (i.e., plague-infected rats and fleas). Since their illnesses went unrecognized and were not appropriately treated, the patients each transmitted their infection to their caregivers, creating this 4-case cluster.

Among the investigation's limitations, we depended on family members' recall for information on deceased patients' symptoms and activities. However, we believe multiple family member interviews and rapid investigation initiation minimized information loss. Another limitation was our inability to culture *Y. pestis* from the surviving patient's sputum, which likely resulted from administration of an antimicrobial drug before sputum collection and suboptimal specimen storage and transport. However, we verified plague infection by laboratory analysis of this sputum sample, including amplification of all 3 targeted plague plasmids by PCR and visualization of numerous bacteria with classic halos of *Y. pestis* by DFA staining. Additionally, the sputum tested strongly positive with both *Y. pestis* antigen dipsticks. Finally, we could not verify all cases through laboratory analysis because 3 case-patients had been buried by the time the outbreak was reported. However, high death rate, fulminant clinical course, laboratory verification for the surviving case, and clinical symptoms were consistent with plague. The concomitant bubonic plague increase and reports of rat deaths provide additional support that plague was endemic during this outbreak.

In conclusion, this investigation illustrates the clinical course of pneumonic plague, contrasts secondary and primary disease, and shows the relatively low communicability of pneumonic plague even with numerous close contacts. This information should guide bioterrorism response planning and the public health response to naturally occurring pneumonic plague outbreaks.

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Host Feeding Patterns of *Culex* Mosquitoes and West Nile Virus Transmission, Northeastern United States

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To evaluate the role of *Culex* mosquitoes as enzootic and epidemic vectors for WNV, we identified the source of vertebrate blood by polymerase chain reaction amplification and sequencing portions of the cytochrome b gene of mitochondrial DNA. All *Cx. restuans* and 93% of *Cx. pipiens* acquired blood from avian hosts; *Cx. salinarius* fed frequently on both mammals (53%) and birds (36%). Mixed-blood meals were detected in 11% and 4% of *Cx. salinarius* and *Cx. pipiens*, respectively. American robin was the most common source of vertebrate blood for *Cx. pipiens* (38%) and *Cx. restuans* (37%). American crow represented <1% of the blood meals in *Cx. pipiens* and none in *Cx. restuans*. Human-derived blood meals were identified from 2 *Cx. salinarius* and 1 *Cx. pipiens*. Results suggest that *Cx. salinarius* is an important bridge vector to humans, while *Cx. pipiens* and *Cx. restuans* are more efficient enzootic vectors in the northeastern United States.

West Nile virus (WNV) has become firmly established in the Western Hemisphere since its discovery in the New York City area in 1999 (*1,2*). The virus has spread at an unprecedented rate throughout the continental United States and to neighboring countries, where it is maintained in an enzootic cycle that involves wild birds and ornithophilic mosquitoes (*3*). To date, 60 mosquito species have been found to be infected with WNV in North America; certain *Culex* spp. appear to be primary vectors, depending on region (*4*). In the northeastern United States, *Culex pipiens*, *Cx. restuans*, and *Cx. salinarius* have been implicated as the principal vectors because they are physiologically competent (*5*), frequently infected with the virus in nature, and closely associated with WNV transmission foci (*6*). However, the precise role that each of these species plays in enzootic transmission among birds or epidemic transmission to humans is not entirely clear.

Entomologic measures of risk may be estimated for different mosquito species by considering their abundance, biting behavior, prevalence of WNV infection, and vector competence. By synthesizing these parameters, Kilpatrick et al. (*7*) estimated that *Cx. pipiens* and *Cx. restuans* were responsible for up to 80% of human infections in New York, whereas *Cx. salinarius* accounted for only 4% of such infections. However, in Connecticut, the abundance of *Cx. salinarius* and prevalence of WNV infection in this species often approach those of *Cx. pipiens* (*6*). Observations in rural and urban sites in New York further indicate that *Cx. pipiens* and *Cx. restuans* are largely ornithophilic, whereas *Cx. salinarius* feeds more frequently on mammals (*8*), which supports the idea of a "bridge vector" role for this species. Nevertheless, collections from New Jersey indicate that mosquitoes of the *Cx. pipiens* complex may readily feed on mammals, including humans (*9*). Further blood meal analysis is required from mosquitoes collected in those habitats that support intense WNV transmission to more fully understand their respective roles as enzootic and epidemic vectors. Such information is vital to the success of any vector control program.

The current research initiative was undertaken to characterize the host-feeding patterns of *Culex* vectors and to evaluate their contribution to enzootic maintenance of WNV in wild bird populations and epidemic transmission to humans. Accordingly, blood-fed mosquitoes were collected from WNV transmission foci in Connecticut and analyzed for host source by sequencing polymerase chain reaction (PCR) amplification products of the vertebrate cytochrome b gene.

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Materials and Methods

Mosquito Collection

Mosquitoes were collected from 31 different sites in 6 counties in Connecticut during a 3-year period (June through October, 2002–2004) as part of a statewide surveillance program (*6*) and a focused trapping effort in Fairfield County (*10*). Most (71%) of the mosquito collection sites were located in densely populated residential locales along the urban/suburban corridor that extends from lower Fairfield and New Haven Counties, where high levels of WNV activity were recorded (Figure, Table 1). Trap sites included parks, greenways, golf courses, undeveloped wood lots, sewage treatment plants, dumping stations, and temporary wetlands associated with waterways. Three trap types were used: a CO_2-baited CDC light trap (John W. Hock Co., Gainesville, FL, USA), a mosquito magnet experimental trap (American Biophysics Corp., East Greenwich, RI, USA), and a CDC gravid mosquito trap (*11*). Typically, traps were operated overnight and retrieved the following morning. Live, adult mosquitoes were transported to the laboratory, where they were promptly identified on chill tables with a stereomicroscope by using descriptive keys (*12*). All mosquitoes with fresh or visible blood remnants were transferred into individual 2-mL tubes labeled according to species, date of collection, and locale and stored at –80°C.

DNA Isolation from Blood-fed Mosquitoes

Mosquito abdomens were removed and reserved for blood-meal analysis with the aid of a dissecting microscope. Each mosquito was dissected individually on a new microscope slide by using flame-sterilized forceps to avoid cross-contamination. DNA was isolated from the abdominal contents of blood-fed mosquitoes individually by using DNA-zol BD, (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's recommendation. Briefly, individual mosquito abdomens were homogenized with heat-sealed pipette tips in 1.5-mL tubes containing DNA-zol BD solution. The homogenates were incubated at room temperature for 5–10 min, mixed, and then centrifuged at 10,000 × *g* for 10 min. DNA was precipitated by adding isopropanol and 3–4 µL Poly Acryl Carrier (Molecular Research Center). The DNA pellet was then washed twice with 75% ethanol, air-dried briefly, reconstituted in TE buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) and stored at –20°C for further analysis.

Blood-meal Analysis

Isolated DNA from the mosquito blood meals served as DNA templates in subsequent PCRs as previously described (*8,9*). PCR primers were based either on a multiple alignment of cytochrome b sequences of avian and

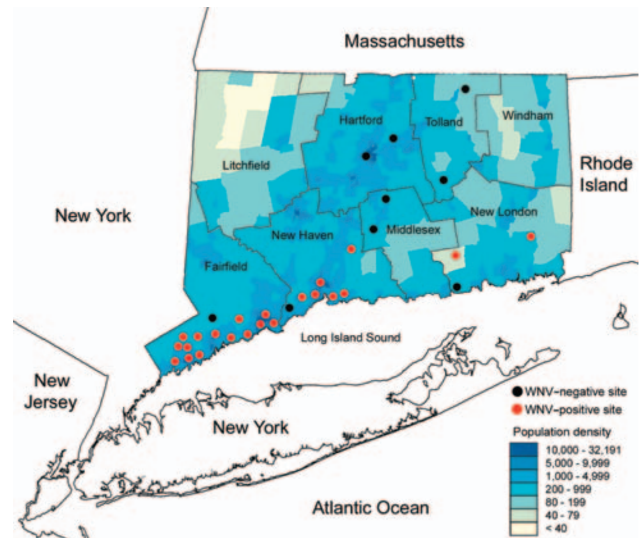

Figure. Geographic distribution of West Nile virus isolations from mosquitoes in relation to human population density and mosquito trapping in Connecticut, 2002–2004. "WNV-positive site" indicates that virus isolations were made from mosquitoes collected from these trapping locations.

mammalian species obtained from GenBank or previously published primer sequences cited in Table 2. All DNA templates were initially screened with avian-a and mammalian-a primer pairs, and the sequences were analyzed (Table 2). In some cases, other primer pairs (avian b, mammalian b and c) were additionally used to resolve ambiguous sequences. A Taq PCR Core Kit (Qiagen, Germantown, MD, USA) was used for all PCRs according to the manufacturer's recommendation. A 50-µL reaction volume was prepared with 3 µL template DNA, 4 µL each primer (0.1–0.5 µmol/L), 5 µL 10× Qiagen PCR Buffer (containing 15 mmol/L $MgCl_2$), 1 µL dNTP mix (10 mmol/L each), 0.25 µL Taq DNA polymerase (1.25 U/reaction) and 32.75 µL water. All PCRs were performed with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) at the ramp speed of 3°C–5°C/s. PCR-amplified products were purified by using QIAquick PCR Purification Kit (Qiagen) and sequenced directly in cycle-sequencing reactions at the Keck Sequencing Facility (Yale University, New Haven, CT, USA) by using the sequencer 3730xl DNA Analyzer (Applied Biosystems). Sequences were annotated by using ChromasPro version 1.22 (Technelysium Pty Ltd., Tewantin, Queensland, Australia) and identified by comparison to the GenBank DNA sequence database (*13*).

The performance of the molecular based assay was validated by isolating DNA from the blood of a number of known vertebrate species and subjecting it to PCR amplification and DNA sequencing. These species included American robin, American crow, black-capped chickadee,

Table 1. No. *Culex* mosquitoes collected for blood-meal analysis from 6 counties in Connecticut, 2002–2004

County	Human population density (per mi^2)	*Culex pipiens*	*Cx. restuans*	*Cx. salinarius*
Fairfield	1,410	195	25	51
New Haven	1,361	17	5	51
Hartford	1,166	–	1	–
Middlesex	420	–	–	2
New London	389	1	1	2
Tolland	332	–	1	–
Total	–	213	33	106

blue jay, button quail, common grackle, eastern tufted titmouse, gray catbird, house sparrow, mourning dove, northern cardinal, sharp-shinned hawk, wood thrush, domestic cat, domestic cow, domestic dog, horse, sheep, white-footed mouse, and white-tailed deer. Similar validation was also conducted with DNA isolated from blood-engorged, laboratory-reared *Aedes aegypti* that fed on guinea pig and button quail. Seasonal changes in the host feeding patterns of *Cx. pipiens* on selected host species were analyzed by χ^2 analysis for trend by using GraphPad Instat version 3.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Blood-meal sources were successfully identified by DNA sequencing from 204 of 213 *Cx. pipiens*, 30 of 33 *Cx. restuans*, and 100 of 106 *Cx. salinarius*. Of 204 *Cx. pipiens* analyzed, 190 (93.1%) contained avian blood only, 5 (2.5%) mammalian, 1 (0.5%) amphibian, and 8 (3.9%) both avian and mammalian blood. Of 100 *Cx. salinarius* analyzed, 36 (36%) contained avian blood only, 53 (53%) mammalian, and 11 (11%) both avian and mammalian blood. All blood meals identified from *Cx. restuans* were avian-derived.

The composition of avian-derived blood meals is shown in Table 3. We identified 27 species of birds as hosts for *Cx. pipiens*; the most frequently identified species were American robin (40.4 % of avian and 37.7% of total), gray catbird (11.1% and 10.4%), and house sparrow (10.6% and 9.9%). Only 1 American crow–derived blood meal was identified for *Cx. pipiens*. Sixteen bird species were identified as hosts for *Cx. restuans*. American robin (36.7%) was the preferred host for *Cx. restuans*, as it was for *Cx. pipiens*, and no crow-derived blood meals were identified. We identified 13 species of birds as hosts for *Cx. salinarius*. The 2 most common avian species were black-capped chickadee (27.7% of avian and 11.7% of total) and American robin (25.5% and 10.8%). More crow-derived blood meals were identified (8.5% and 3.6%) in this mosquito species.

A seasonal shift from American robins to other avian species was noted with *Cx. pipiens* (Table 4). The χ^2 test for linear trend showed that the proportion of American robin–derived blood meals decreased from June until October ($p<0.0001$). In June, 62.4% of all avian-derived blood meals were obtained from American robins, and this percentage declined to 26.7% in July and 38.9% in August. By September, 25.7% of the avian-derived blood meals were obtained from gray catbirds and 20.0% from mourning doves, while none was identified as being from American robins.

An analysis of the mammalian blood meal sources for *Cx. pipiens* and *Cx. salinarius* is shown in Table 5. We identified 10 host species for *Cx. salinarius* and 7 for *Cx. pipiens*. White-tailed deer (*Odocoileus virginianus*) was the most frequently identified host for *Cx. salinarius* (67.2% of mammalian and 38.7% of total). Human-derived blood meals were identified from 2 *Cx. salinarius* and 1 *Cx. pipiens*.

Table 2. Sequences of primers, length of amplification products, and thermal cycling conditions used in polymerase chain reactions for blood-meal analysis

			Cycling condition			
Primer name	Sequence	Product (bp)	Denaturation	Annealing	Extension	No. cycles
Avian a	GAC TGT GAC AAA ATC CCN TTC CA (f)* GGT CTT CAT CTY HGG YTT ACA AGA C (r)	508	94°C (30 s)	60°C (50 s)	72°C (40 s)	36
Avian b	CCC TCA GAA TGA TAT TTG TCC TCA (f)† CCT CAG AAK GAT ATY TGN CCT CAK GG (r)	515	95°C (1 min)	58°C (1 min)	72°C (1 min)	35
Mammalian a	CGA AGC TTG ATA TGA AAA ACC ATC GTT G (f) TGT AGT TRT CWG GGT CHC CTA (r)	772	94°C (30 s)	55°C (45 s)	72°C (1.5 min)	36
Mammalian b	GCG TAC GCA ATC TTA CGA TCA A (f) CTG GCC TCC AAT TCA TGT GAG (r)	195	95°C (1 min)	54°C (1 min)	72°C (1 min)	32
Mammalian c	CCA TCC AAC ATC TCA GCA TGA TGA AA (f) GCC CCT CAG AAT GAT ATT TGT CCT CA (r)	395	95°C (1 min)	55°C (1 min)	72°C (1 min)	32

*Reference (*39*).
†Reference (*40*).

Table 3. Number and percentage of avian blood meals identified from *Culex* mosquitoes collected in Connecticut, 2002–2004

	*Culex pipiens**			*Cx. restuans*		*Cx. salinarius†*		
Avian species	No.	% of avian (n = 198)	% of total (n = 212)	No.	% (n = 30)	No.	% of avian (n = 47)	% of total (n = 111)
American robin (*Turdus migratorius*)	80	40.4	37.7	11	36.7	12	25.5	10.8
Gray catbird (*Dumetella carolinensis*)	22	11.1	10.4	2	6.7	–	–	–
House sparrow (*Passer domesticus*)	21	10.6	9.9	–	–	5	10.6	4.5
European starling (*Sturnus vulgaris*)	14	7.1	6.6	1	3.3	1	2.1	0.9
Mourning dove (*Zenaida macroura*)	13	6.6	6.1	2	6.7	3	6.4	2.7
Black-capped chickadee (*Poecile atricapilla*)	9	4.5	4.2	1	3.3	13	27.7	11.7
Common grackle (*Quiscalus quiscula*)	8	4.0	3.8	2	6.7	–	–	–
Wild turkey (*Meleagris gallopavo*)	6	3.0	2.8	1	3.3	–	–	–
Northern cardinal (*Cardinalis cardinalis*)	4	2.0	1.9	2	6.7	–	–	–
House finch (*Carpodacus mexicanus*)	3	1.5	1.4	–	–	1	2.1	0.9
Barn swallow (*Hirundo rustica*)	2	1.0	0.9	–	–	–	–	–
American crow (*Corvus brachyrhynchos*)	1	0.5	0.5	–	–	4	8.5	3.6
Sharp-shinned hawk (*Accipiter striatus*)	1	0.5	0.5	1	3.3	1	2.1	0.9
Brown-headed cowbird (*Molothrus ater*)	1	0.5	0.5	1	3.3	1	2.1	0.9
Canada goose (*Branta canadensis*)	1	0.5	0.5	1	3.3	1	2.1	0.9
Wood thrush (*Hylocichla mustelina*)	1	0.5	0.5	1	3.3	–	–	–
Red-winged blackbird (*Agelaius phoeniceus*)	1	0.5	0.5	–	–	–	–	–
Cedar waxwing (*Bombycilla cedrorum*)	1	0.5	0.5	–	–	–	–	–
Red-tailed hawk (*Buteo jamaicensis*)	1	0.5	0.5	–	–	–	–	–
Green heron (*Butorides virescens*)	1	0.5	0.5	–	–	–	–	–
Rock dove (*Columba livia*)	1	0.5	0.5	–	–	–	–	–
Song sparrow (*Melospiza melodia*)	1	0.5	0.5	–	–	–	–	–
Indigo bunting (*Passerina cyanea*)	1	0.5	0.5	–	–	–	–	–
House wren (*Troglodytes aedon*)	1	0.5	0.5	–	–	–	–	–
Willow flycatcher (*Empidonax traillii*)	1	0.5	0.5	–	–	–	–	–
Black-and-white warbler (*Mniotilta varia*)	1	0.5	0.5	–	–	–	–	–
Northern waterthrush (*Seiurus noveboracensis*)	1	0.5	0.5	–	–	–	–	–
Wood duck (*Aix sponsa*)	–	–	–	–	–	2	4.3	1.8
Prairie warbler (*Dendroica discolor*)	–	–	–	1	3.3	–	–	–
Mallard (*Anas platyrhynchos*)	–	–	–	–	–	1	2.1	0.9
Northern oriole (*Icterus galbula*)	–	–	–	1	3.3	–	–	–
Black-crowned night heron (*Nyctiocorax nyctiocorax*)	–	–	–	–	–	2	4.3	1.8
Rose-breasted grosbeak (*Pheucticus ludovicianus*)	–	–	–	1	3.3	–	–	–
Blue-headed vireo (*Vireo solitarius*)	–	–	–	1	3.3	–	–	–

*Includes 8 specimens from which double-blood meals were identified.
†Includes 11 specimens from which double-blood meals were identified.

Discussion

Our analysis on the blood-feeding behavior of *Culex* mosquitoes provides insight into their relative roles as enzootic and epidemic vectors of WNV in this region of the northeastern United States. We found that *Cx. pipiens* and *Cx. restuans* predominantly feed on avian hosts and focus their feeding activity on several key bird species that can support WNV transmission, in particular, American robins, gray catbirds, and house sparrows. By contrast, we found that *Cx. salinarius* feeds more opportunistically than *Cx. pipiens* and *Cx. restuans* and includes a relatively high proportion of mosquitoes with mixed blood meals from both avian and mammalian sources. This finding suggests that *Cx. salinarius* serves as a bridge vector by transferring WNV from viremic birds to mammalian hosts.

The preponderance of WNV isolations obtained from *Cx. pipiens* in surveillance activities conducted over the last 6 years (*6,10,14,15*) clearly incriminates this species as the predominant mosquito vector in this region. However, while enzootic transmission to birds is strongly supported by a number of host-preference studies on regional populations (*8,9,16–18*), no consensus has been reached on the role of *Cx. pipiens* in epidemic transmission of WNV to humans in the northeastern United States. Apperson et al. (*9*) recently identified mammalian-derived blood meals in 38% of blood-fed *Cx. pipiens*, 10.8% of which were human-derived (≈2.5% overall), collected from New Jersey. This finding led these researchers to conclude that *Cx. pipiens* was likely an epidemic vector in that region. This interpretation was viewed as consistent with

Table 4. Monthly prevalence of avian-derived blood meals in *Culex pipiens*

Month	Total avian	American robin, n (%)	Gray catbird, n (%)	House sparrow, n (%)	Mourning dove, n (%)	European starling, n (%)	Other species, n (%)
June	93	58 (62.4)	2 (2.2)	4 (4.3)	–	12 (12.9)	17 (18.3)
July	30	8 (26.7)	2 (6.7)	9 (30.0)	3 (10.0)	2 (6.7)	6 (20.0)
August	36	14 (38.9)	6 (16.7)	4 (11.1)	3 (8.3)	–	9 (25.0)
September	35	–	9 (25.7)	3 (8.6)	7 (20.0)	–	16 (45.7)
October	4	–	3 (75.0)	1 (25.0)	–	–	–
Total	198	80	22	21	13	14	48

the incidence of human cases in 3 densely populated urban areas of Connecticut in 2002, where most viral isolations (78%) were from *Cx. pipiens* (*6*). Kilpatrick et al. (*7*), integrating WNV testing data from New York from 2000 to 2003 with information on mosquito abundance, infection prevalence, vector competence, and biting behavior, further suggested that *Cx. pipiens* and *Cx. restuans* were responsible for up to 80% of human infections in that region. However, the validity of their conclusions was based on the identification of mammalian-derived blood in ≈19% of these 2 species and the assumption that humans were also included. Our analysis of blood meals from wild-caught female *Cx. pipiens* from established WNV foci in Connecticut is inconsistent with this supposition, as this species shows a strong tendency for avian blood and little inclination for mammalian hosts, including humans. We, therefore, conclude that while *Cx. pipiens* may occasionally feed on humans, it may not be the predominant vector of WNV to humans in our region of the northeastern United States. This finding is compatible with the lack of any mammalian-derived blood meals in blooded *Cx. pipiens* collected from suburban locales in nearby Westchester County, New York (*9*), but contrasts sharply with a recent study conducted in Delaware, where 69% of the blood meals taken by *Cx. pipiens* were from large mammals (*19*), which suggests a difference in host preference from more southern regions of its range.

Examination of the blood-fed mosquitoes in the present study showed an exclusively ornithophilic nature of *Cx. restuans*; all analyzed blood meals were from avian species. These findings were consistent with prior host preference studies (*8,9,16,20*) and strongly support the view that this predominant "early season" species is most likely involved in initiation and amplification of WNV transmission among wild birds and rarely, if ever, feeds on humans in this region. This finding differs from a recent blood-meal analysis by Gingrich and Williams (*19*), who found that a limited number (n = 9) of *Cx. restuans* from Delaware were highly mammalophilic (9:1 mammal-to-bird ratio). However, they concluded that this species was still primarily an enzootic vector since they never collected it in human landing collections.

Our findings regarding the blood-feeding patterns of *Cx. salinarius* reinforce those of previous studies (*18,20–25*) and indicate that this species feeds indiscriminately on both birds and mammals, including humans. By using separate PCR primer pairs for different vertebrate classes, we find that 11% of *Cx. salinarius* acquired blood meals from both avian and mammalian sources, versus ≈4% for *Cx. pipiens*. We cannot say whether all or most of these double-source blood meals represent multiple feeding episodes during the same gonotrophic cycle or the detection of residual DNA from a prior egg-laying cycle. However, mixed-source blood meals have been reported for a number of *Culex* species by using different methods for blood-meal identification (*8,16*). Regardless, our findings indicate that a relatively large fraction of the *Cx. salinarius* population readily feeds on both birds and mammals, which is a necessary condition for epidemic transmission to humans. The opportunistic feeding pattern of *Cx. salinarius*, in conjunction

Table 5. Number and percentage of mammalian blood meals taken by *Culex* mosquitoes collected in Connecticut, 2002–2004

Species	*Culex pipiens**			*Cx. salinarius*†		
	No.	% of mammal (n = 13)	% of total (n = 212)	No.	% of mammal (n = 64)	% of total (n = 111)
White-tailed deer (*Odocoileus virginianus*)	4	30.8	1.9	43	67.2	38.7
Gray squirrel (*Sciurus carolinensis*)	3	23.1	1.4	–	–	–
Northern raccoon (*Procyon lotor*)	2	15.4	0.9	2	3.1	1.8
Human (*Homo sapiens*)	1	7.7	0.5	2	3.1	1.8
Virginia opossum (*Didelphis virginiana*)	1	7.7	0.5	4	6.2	3.6
Dog (*Canis familiaris*)	–	–	–	4	6.2	3.6
Cat (*Felis catus*)	1	7.7	0.5	3	4.7	2.7
Eastern cottontail (*Sylvialagus floridanus*)	1	7.7	0.5	3	4.7	2.7
Horse (*Equus caballus*)	–	–	–	1	1.6	0.9
Striped skunk (*Memphitis memphitis*)	–	–	–	1	1.6	0.9
Brown rat (*Rattus norvegicus*)	–	–	–	1	1.6	0.9

*Includes 8 specimens from which double-blood meals were also identified.
†Includes 11 specimens from which double-blood meals were also identified.

with its physiologic competence to transmit WNV (*26*), high infection rates in nature (*10,14,15*), and seasonal distribution that overlaps with human cases (*6*), all indicate that this species is a bridge vector of WNV to humans in the northeastern United States.

White-tailed deer were the single most important source of blood for *Cx. salinarius* in our study, which supports similar findings from New Jersey (*9,18*). The apparent affinity of *Cx. salinarius* for deer over other mammalian hosts is likely a function of deer's availability, as they are the most abundant large mammals in the region after humans. The role of deer in the ecology and transmission dynamics of WNV is unknown. Seroprevalence of WNV antibodies was 0%–6% among hunter-killed deer from New Jersey in 2001 (*27*), which suggests infrequent exposure to WNV relative to avian hosts, but frequency of exposure is still greater than that in humans (*28*). Widespread abundance of deer could be zooprophylactic by diverting feeding from avian amplifying hosts to deer. This possibility merits further study.

Several avian hosts are highly susceptible to WNV infection and can support viremia sufficient to infect culicine vectors. Reservoir competence values expressed as the duration and magnitude of infectious-level viremia were evaluated for 25 bird species and shown to be highest for passerine birds, including the blue jay, common grackle, house finch, American crow, house sparrow, and American robin (*29*). Field data further implicate a few species as reservoir hosts in northeastern United States on the basis of their exposure to WNV. When abundance and seroprevalence data were combined, house sparrows were estimated to be the most commonly infected bird species in New York City (*30,31*). These findings, combined with reservoir competence data, suggest that house sparrows are amplifying hosts in urban locales; however, other resident bird species, such as the northern cardinal, house finch, and gray catbird, were also frequently exposed to WNV (*30*). We show that in Connecticut, *Cx. pipiens* and *Cx. restuans* acquire blood meals predominately from American robins, implicating this species as a reservoir host for WNV. American robins are moderately competent and develop infectious-level viremia for a duration of ≈3 days (*29*). This species is most abundant in Connecticut from early spring to midsummer (*32*). Therefore, they may support more early- to mid-season (June–August) amplification of the virus. Our findings of a seasonal shift in *Cx. pipiens* from American robins to other avian species support this hypothesis.

We found that *Cx. pipiens* and *Cx. restuans* rarely fed upon American crows, despite their abundance (*32*) and high death rate from WNV infection throughout the region (*33–35*). Similar findings were reported from *Cx. pipiens*–complex mosquitoes collected from New York (*8*) and New Jersey (*9*). This finding suggests that American crows may also acquire WNV through other means in addition to mosquito transmission. American crows are susceptible to WNV infection by oral ingestion of the virus in aqueous solution and by eating infected bird carcasses (*29*). These birds are aggressive nest raiders and, therefore, could also acquire WNV infection by eating infected nestling birds. Transmission could also occur directly from bird to bird, as has been demonstrated in laboratory settings for this and other species (*29,36,37*). Our findings indicate that American crows may not be the primary amplifying hosts for infecting *Culex* mosquitoes with WNV in this region of the northeastern United States. Alternatively, we find that other common birds, including American robins, gray catbirds, and house sparrows, may play a greater role in supporting enzootic transmission.

Our PCR-based method took advantage of the conservation and diversity of mitochondrial sequences in identifying the source of vertebrate blood from mosquitoes. Mitochondrial DNA is a useful marker in phylogenetic studies and molecular systematics because of its maternal inheritance, haploid nature, and rapid rate of evolution (*38*). The cytochrome b gene, in particular, has successfully been used to identify taxonomic groups to the subspecies level and these sequences are publicly accessible from a wide array of different bird and mammal species in the GenBank database. By sequencing portions of the cytochrome b gene, we unambiguously identified the blood-meal source to the species level, which represents an improvement in sensitivity and specificity over earlier analyses.

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Dr Molaei is a postdoctoral scientist at the Connecticut Agricultural Experiment Station. His current research interests include epidemiology of WNV, in particular mosquito-host interactions and host feeding patterns of mosquito vectors.

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Identifying and Quantifying Genotypes in Polyclonal Infections due to Single Species

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Simultaneous infection with multiple pathogens of the same species occurs with HIV, hepatitis C, Epstein-Barr virus, dengue, tuberculosis, and malaria. However, available methods do not distinguish among or quantify pathogen genotypes in individual patients; they also cannot test for novel insertions and deletions in genetically modified organisms. The strategy reported here accomplishes these goals with real-time polymerase chain reaction (PCR) and capillary electrophoresis. Real-time PCR with allotype-specific primers defines the allotypes (strains) present and the intensity of infection (copy number). Capillary electrophoresis defines the number of genotypes within each allotype and the intensity of infection by genotype. This strategy can be used to study the epidemiology of emerging infectious diseases with simultaneous infection by multiple genotypes, as demonstrated here with malaria. It also permits testing for insertions or deletions in genetically modified organisms that may be used for bioterrorism.

Simultaneous infection with multiple pathogens of the same species occurs in human patients with HIV, hepatitis C, Epstein-Barr virus, dengue, tuberculosis, and malaria (*1–7*). However, available laboratory methods do not distinguish among pathogen genotypes in samples from individual patients. They do not permit the identification or quantitation of genotypes in samples with multiple pathogens of the same species, or the identification of size polymorphisms produced by insertions and deletions.

Conventional polymerase chain reaction (PCR) with agarose gel electrophoresis permits the identification of pathogen allotypes (strains) in human blood and tissue and an assessment of the sizes of their amplicons but does not define allotype copy number or genotype copy number. Real-time PCR permits identification and quantitation of allotypes (*8,9*) but does not permit the identification of genotypes within allotypes.

From the epidemiologic perspective, a molecular strategy to define the allotypes and genotypes of human pathogens and their copy numbers would permit one to study the dynamics of simultaneous infection with multiple genotypes in ways that have been impossible. For example, this knowledge could be used to identify novel genotypes (size polymorphisms) resulting from insertions and deletions at polymorphic loci.

From the bioterrorism perspective, a strategy to identify size polymorphisms (insertions and deletions) in critical regions of pathogen genomes would be of immense value. This information could be used to test for deletions in regulatory (suppressor) regions and for the insertion of new genes in regions controlled by strong promoters. Available methods do not permit the rapid identification of size polymorphisms within allotypes or the quantitation of individual pathogen genotypes.

To address this challenge, we used real-time PCR and capillary electrophoresis. Real-time PCR with allotype-specific primers permits one to define the allotypes present and their copy number (*8,9*). Capillary electrophoresis permits one to define individual genotypes within allotypes and genotype copy number. The combination of real-time PCR and capillary electrophoresis also permits the identification of insertions and deletions in potentially critical regions of pathogen genomes.

Materials and Methods

Collection of Patient Samples and Isolation of Pathogen DNA

Using fingersticks, filter paper blot samples (S & S #903 Blood Collection Cards, Schleicher & Scheuell Bioscience, Keene, NH, USA) containing 50 µL of blood

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were obtained from persons with *Plasmodium falciparum* infection in Mali. These samples were obtained in a prospective study of asymptomatic *P. falciparum* infection in the village of Missira (160 km northwest of Bamako), after review and approval by the University of Bamako Institutional Review Board (IRB) in Bamako and the Tulane University IRB in New Orleans. Before obtaining informed consent from the participants (after IRB reviews and approvals), the protocol was reviewed with the chief and elders of the village and the women's council. After those additional reviews and approvals, informed consent was obtained from persons ≥18 years of age and from the parents and guardians of children ≤17 years of age before obtaining blood samples. DNA was isolated from filter paper blots and blood samples by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA).

Control template DNA was obtained from cultured parasites (*10*) by using the QIAamp DNA Blood Mini Kit (Qiagen). Cloned isolates used as controls for the 4 known allotypes of the polymorphic block 2 region of merozoite surface protein 1 (*msp1*) were Indochina I/CDC for MAD20, Haiti 135 for K1, 7G8 for RO33, and OK/JC 5 for MAD20/RO33 hybrid allotype parasites (*11–13*). DNA template concentrations were estimated from standard curves by plotting the fluorescence of 5 DNA standards with concentrations of 1 µg/mL (1,000 ng/ml), 100 ng/mL, 10 ng/mL, 1 ng/mL, and 0 ng/mL (blank or negative control) vs. the $\log_{10}$ of template DNA concentration by using PicoGreen dye (Molecular Probes, Eugene, OR, USA) with the VersaFluor fluorometer (Bio-Rad, Hercules, CA, USA).

Primer and Probe Design for Real-time PCR

Primers and probes were designed by using the Beacon Designer software, version 2.03, Premier (Biosoft International, Palo Alto, CA, USA) (available from http://www.premierbiosoft.com/molecular_beacons/), in combination with manual manipulation. The primers and probes used to amplify the K1, MAD20, RO33, and hybrid (MAD20/RO33) allotypes of the block 2 region of *msp1* and the internal control gene (erythrocyte-binding antigen 175, *eba175*) in *P. falciparum* are listed in Table 1, together with information on fluorophores, melting temperatures, final reactant concentrations, and the observed ranges of amplicon sizes. Unlabeled primers and fluorophore-labeled probes were obtained from Integrated DNA Technologies (Coralville, IA, USA), LUX-labeled primers from Invitrogen (Carlsbad, CA, USA); and the Cy5-labeled probe for *eba175* from Biosearch Technologies (Novarto, CA, USA).

Real-time PCR Amplification of Pathogen DNA

Real-time PCR was performed with the iCycler (Bio-Rad) by using the amplification conditions described below (Table 1) with a 2× multiplex-specific master mix (Qiagen) and 3-µL aliquots of template DNA. Reaction mixtures were supplemented with 2.5 U recombinant Taq polymerase (Invitrogen) and subjected to an initial denaturation at 95°C for 15 min, followed by 45 cycles of denaturation at 94° C for 30 s, annealing at 53°C for 90 s, and extension at 72°C for 90 s. Fluorescence measurements were obtained during the annealing step with TaqMan probes (K1 and MAD20/RO33 hybrid allotypes, and the *eba175* internal control), and during the elongation step with LUX primers (MAD20 and RO33 allotypes). Each sample was tested in quadruplicate. Two samples were used to define the allotypes present and their copy number with the iCycler; the other 2 samples were removed from the iCycler during the exponential (logarithmic) stage of

Table 1. Primers and probes used for real-time polymerase chain reaction*

Primer/probe sequence	Sizes (bp)	T_m (°C)	Fluorophore, quencher	Final reactant concentration (nmol/L)
Parasites with K1 and hybrid sequences in block 2 of *msp1*				
K1F 5′-AGGTGCAAGTGCTCAAAGTG-3′	108–171	50.3	Texas Red, BHQ-2	100
K1R 5′-CACCAGATGAAGTATTTGAACG-3′		49.2		100
PROBE: 5′-AAGTGGTACAAGTCCATCATCTCGT-3′		54.9		300
Hybrid F 5′-GAAGGAACAAGTGGAACAGC-3′	96–168	48.7	Cy5, BHQ-2	200
Hybrid R 5′-GCAGCACCTGGAGATCTTATA-3′		48.8		200
PROBE: 5′-TTCACTTATTTCCCCATGAGCCCC-3′		54.2		400
Primers for positive control (EBA175)				
EBA175 F 5′-GGTTATTCAACTAAGGCAGAA-3′	95	46.0	Cy 5, BHQ-3	100
EBA175 R 5′-TCCACCATTCTTTTCTAAAATTTT-3′		50.6		100
PROBE: 5′-TCATTTCCCATAGCAAGATGTCC		60.0		100
LUX primers (MAD 20, RO33)				
MAD20 F 5′-AATGAAGGAACAAGTGGAAC-3′	52–205	48.7	FAM	200
MAD20 R 5′-GAATTATCTGAAGGATTTGTACG-3′		47.4	(reverse)	200
RO33 F 5′-GCAGATGCTGTAAGTACTCAA-3′	148	44.8	JOE	200
RO33 R 5′-GCAGCACCTGGAGATCTTATA-3′		44.8	(forward)	200

*msp1, merozoite surface protein 1, EBA175, erythrocyte-binding antigen 175; T_m, melting temperature.

amplification for capillary electrophoresis to define the genotypes present and genotype copy number.

Template Specificity and Optimization of Multiplex PCR

Template specificity was tested for each primer probe set with the 4 control template DNAs (from Indochina I, Haiti 135, 7G8 and hybrid MAD20/RO33 parasites). Amplicons of the expected sizes were obtained with matched template and primer probe sets; no amplicons were obtained with unmatched template and primer probe sets. Negative controls likewise yielded no amplicons. DNA extracted from specimens without parasites was used to control for primer-primer and primer-probe interactions, and other potential causes of false-positive PCR results. After establishing specificity, the reaction conditions were optimized by defining the efficiencies of each primer probe set using a series of 10-fold dilutions with each control template DNA. These efficiencies were then matched to the efficiencies obtained with the multiplex PCR to adjust the final primer and probe concentrations so the efficiencies of the multiplex PCR matched those of the individual PCRs.

PCR Amplification and Allotype Quantitation

Standard curves were generated by using 10-fold dilutions of template DNA (3-µL aliquots) from each of the control parasites to estimate the initial copy numbers of the 4 allotypes in each sample. The standard curves (regression lines) for each allotype, the resulting reaction efficiencies, threshold cycle (C_T) values and estimates of initial copy numbers were calculated by using the iCycler Software (Bio-Rad).

Capillary Electrophoresis and Genotype Quantitation

To estimate amplicon size (base pairs) and copy number for each genotype, 2 replicates were removed from the iCycler for each sample during the logarithmic amplification stage, as determined by real-time relative fluorescence unit (RFU) data, and stopped with 0.5 mol/L EDTA. For each reaction, two 1-µL aliquots of the real-time PCR reaction mixture were loaded onto a DNA 500 Lab Chip (Agilent Technologies, Waldbronn, Germany) and run on the Bioanalyzer 2100 (Agilent Technologies), according to the manufacturer's instructions. With capillary electrophoresis using the DNA 500 Lab Chip, a linear relationship was shown between amplicon size and elution time ($r^2 \geq 0.998$, $p < 0.001$ for amplicons from 25 bp to 400 bp; data not shown). The copy numbers for each genotype were calculated from the molarities provided by the Agilent software. These calculations are based on the observation that the concentration of each amplicon is proportional to its peak area on the electropherogram.

Results

Real-time PCR To Identify Pathogen Allotypes

Real-time PCR with allotype-specific primers permits the amplification of individual allotypes in specimens from infected human subjects (first 3 columns of Table 2, and Figure 1, panel A). Based on control specimens containing only 1 allotype and on negative controls, this strategy is specific. Based on filter paper blots for specimens containing ≥100 parasites/µL, it is sensitive. However, real-time PCR with allotype-specific primers does not distinguish among (identify) genotypes within allotypes (Figure 1). This is because real-time PCR cannot identify size polymorphisms, whether they result from natural events such as the spontaneous addition and deletion of tripeptide repeats in malaria parasites (*14*) or deliberately malevolent manipulation of microorganisms in the laboratory as potential agents of bioterrorism.

Optimization of Real-time PCR

Estimates of efficiency (the degree to which replication increases the number of amplicons by the expected 2-fold increment [100% efficiency] in each cycle) indicate that the efficiency of the real-time PCR assays performed in these studies was excellent (90%–100%). In addition, efficiencies of reactions in multiplex did not differ significantly from individual reaction efficiencies or from other reaction efficiencies in multiplex.

Reproducibility of Copy Number (Threshold Cycle, C_T) Estimates

Data for estimates of copy number were based on the amplification of block 2 of *msp1* from *P. falciparum* malaria parasites (Table 2). The reproducibility of C_T estimates was examined separately for exemplary 93- and 154-bp amplicons, and found to have means of 27.99 and 28.62 cycles, respectively, with standard deviations of 0.34 and 0.13 cycles (i.e., coefficients of variation [CVs] of 1.2% and 0.5% for these 2 amplicons, n = 10 for each).

Capillary Electrophoresis To Identify Pathogen Genotypes

In contrast to real-time PCR, which identifies only allotypes, capillary electrophoresis identifies genotypes within allotypes (based on size polymorphisms) in samples from persons (Figure 1, panels B–D). Across participants (in groups of samples), this method permits one to identify the spectrum (range) of genotypes in the population (data for samples from 10 persons infected with *P. falciparum* are presented as an example in Figure 2 and Table 3). The reproducibility of capillary electrophoresis is sufficient to separate amplicons that differ by ≥5 bp. This conclusion was based on a comparison of amplicons containing 148

Table 2. Distribution of allotypes and genotypes in 1 blood sample*

Allotype	Amplicon size, bp (no. repeats)	Allotype copy no.	Proportions of individual genotypes (%)	Genotype copy no.
MAD20	184 (15)	1.38×10^3	100	1.38×10^4
K1	106 (9)	1.53×10^4	19	2.90×10^3
K1	124 (11)		61	9.30×10^3
K1	133 (12)		7	1.10×10^3
K1	151 (14)		13	1.99×10^3
Hybrid	159 (7)	4.67×10^5	100	4.67×10^5
RO33	NA	NA	0	0

*NA, not available.

and 153 bp with elution times on electropherograms of 60.51 and 61.11 s, respectively (Figure 3).

Reproducibility of Genotype Copy Number Estimates

Based on the electropherograms, the reproducibility of peak area measurements and estimates of genotype copy number was excellent. CVs varied from 0.13% to 0.45% for amplicon concentrations between 10 nmol/L and 80 nmol/L (n = 12 replicates at each of 4 template concentrations of a 95-bp amplicon from 10 nmol/L to 80 nmol/L, data not shown).

Real-time Fluorescence in Relation to Peak Area

The slopes of increasing fluorescence based on real time PCR with the iCycler were indistinguishable from the increasing peak areas on the electropherogram (Figure 4, panels A and B, slopes of 0.2252 and 0.2223, $p > 0.5$). The similarity of these slopes (based on different parameters) indicates that increases in RFUs are directly proportional to increases in amplicon concentration (molarity). This result permits one to extrapolate from allotype copy number to genotype copy number based on peak area.

Field Samples from Persons with Polyclonal Infections

Three of the 4 known *P. falciparum* allotypes have size polymorphisms within block 2 of *msp1*. K1, MAD20, and hybrid MAD20/RO33 allotype parasites have size polymorphisms because they contain tripeptide repeats within block 2 of *msp1*; RO33 does not have size polymorphisms because it does not have tripeptide repeats (*15*). These size polymorphisms are evident for K1 in a sample from a single person (Table 2) and for K1, MAD20, and hybrid MAD20/RO33 parasites in samples from 10 persons (Figure 2 and Table 3).

Discussion

Simultaneous Infection and Detection of Genetically Modified Organisms

Studies by a number of investigators have shown that simultaneous infection with multiple pathogens (genotypes) of the same species occurs in patients with HIV, hepatitis C, Epstein-Barr virus, dengue, tuberculosis, and malaria (*1–7*) and have identified deletions and insertions (genotypes) due to tandem repeats in cytomegalovirus (*15*). Because pathogen genotypes based on insertions and deletions are common, the strategy reported here is potentially applicable to all microbial human pathogens. This complexity of infection is likely to be important in the pathogenesis and transmission of many emerging infectious diseases. For example, epidemiologically and clinically meaningful events such as severe disease and antimicrobial drug resistance are likely to be driven by competition among pathogen genotypes in vivo (by the virulence and antimicrobial susceptibility/resistance determinants of the predominant genotypes) and may also affect transmission.

In addition to block 2 of *msp1* in *P. falciparum*, other examples of natural sequence variation detectable by using real-time PCR and capillary electrophoresis (variations ≥5 nucleotides/bp) include duplications and deletions in the 3′ noncoding regions (NCRs) of dengue (*16*) and yellow fever (*17*) and insertions and deletions in the *env* gene of HIV (*18,19*). For *Mycobacterium tuberculosis*, examples include variation in the tandem repeats within *IS*6110 (*20*), variable numbers of tandem repeats (VNTRs) (*21*), and genomic deletions (*22*). For select agents, examples include variation in VNTRs (multiple locus VNTR analysis) in *Bacillus anthracis* (*23,24*), similar differences in *Yersinia pestis* (*25*), and insertions, deletions, and variation in the inverted terminal repeat region and the coding region of the smallpox virus (*26,27*) (Table 4).

In addition, disease-producing agents may be modified in the laboratory to increase their virulence or to introduce antimicrobial drug resistance for bioterrorist events (*28–30*). However, available methods are inadequate to rapidly diagnose and quantitate simultaneous infection with multiple pathogens (genotypes) of the same species or identify insertions and deletions in critical regions of pathogen genomes. The results reported here provide a strategy to address these issues based on real-time PCR and capillary electrophoresis.

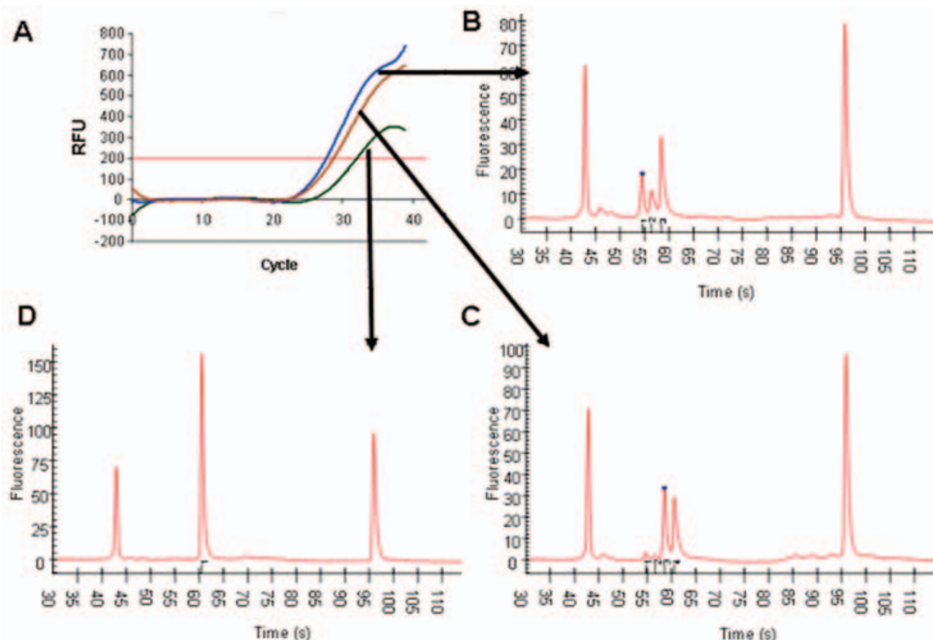

Figure 1. Use of capillary electrophoresis to identify multiple genotypes within single allotypes amplified by real-time polymerase chain reaction. Panel A shows the relative fluorescence values for 3 samples from infected patients by using primers specific for the K1 allotype of merozoite surface protein 1 (*msp1*). Panels B, C, and D show that those samples contained 3, 4, and 1 different K1 genotype parasites, respectively, identified by amplicons of 106, 124, and 142 bp (panel B), 105, 124, 142, and 160 bp (panel C), and 160 bp (panel D), respectively. The first and last peaks on each electropherogram are the 15- and 600-bp standard markers used to define the sizes of the unknown amplicons.

Real-time PCR To Identify and Quantitate Pathogen Allotypes

As demonstrated here and elsewhere, allotype-specific primers permit one to identify the pathogen allotypes in a specimen (*1–7*). In addition, real-time PCR may be used to quantitate the numbers of microorganisms in a specimen. Because the relationship between the number of cycles necessary to reach the C_T and the $\log_{10}$ of copy number is linear, real-time PCR can be used to estimate the initial amount of template DNA (copy number) (*8,9*).

Capillary Electrophoresis To Identify and Quantitate Pathogen Genotypes

In contrast to real-time PCR (in which all amplicons [genotypes] are examined together in the same well once each cycle), capillary electrophoresis detects the amplicons from each genotype as they pass a fluorescence or absorbance detector. This is accomplished by separating dsDNA amplicons based on their size (base pairs) by using a charged electrical field to drive the dsDNA polyanions to the detector at the anode. Because this separation is driven by the ratio of the electrical driving force to the mass of each amplicon, the rate of movement to the anode is inversely proportional to mass (size in base pairs). Thus, smaller amplicons travel faster and have shorter retention times on the electropherogram (Figure 1, panels B–D).

Detection of Artificial-size Polymorphisms

The results reported here demonstrate that capillary electrophoresis is sufficiently sensitive to detect insertions and deletions ≥5 bp in size. This finding means that capillary electrophoresis is more than sufficiently sensitive to detect biologically significant insertions and deletions in genetically modified organisms (*23–30*). Thus, it provides an open-ended strategy to test for genetically modified organisms, by testing for size polymorphisms at critically important sites in the pathogen genome, e.g., at sites related to pathogenicity (virulence) or antimicrobial resistance.

Advantages, Limitations, and Potential Pitfalls

The advantages of real-time PCR followed by capillary electrophoresis are that it can be performed without waiting days or weeks for cultures to grow and that it detects pathogens that do not grow in conventional culture media or under standard conditions (*31*). In addition, as noted above (*14*), sequence information is enormously helpful in

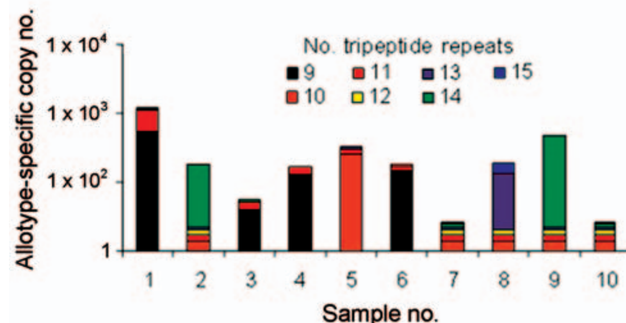

Figure 2. Copy numbers for genotypes of the K1 allotype in 10 field samples. Distribution of K1 genotypes within the 8 patients whose samples yielded amplicons with K1-specific primers (Table 4). These results indicate that most infected persons had ≥2 allotypes. In addition, persons with K1 allotype parasites had a high degree of genotypic complexity, that is, capillary electrophoresis showed up to 4 distinct K1 genotypes in the blood of individual patients at the same time.

Table 3. Copy number for amplification of *msp1* allotypes in field samples*

Field sample	Allotype			
	MAD20	RO33	K1	Hybrid
1	1,380	0	15,300	467,000
2	0	361,000	342	0
3	813	0	25.53	0
4	138.4	0	261.4	0
5	25.65	0	1,090	101
6	6.71	0	323.7	29.7
7	143	0	22.6	75.7
8	548	0	361	0
9	717	0	2,320	11.9
10	2,330	226	44.6	178

*Distribution of allotype-specific copy numbers for samples from 10 subjects infected with *Plasmodium falciparum*. Both MAD20 and K1 allotype parasites were present in 8 of the 10 subjects, MAD/RO hybrid allotypes were present in 4, and RO33 was present in 1.

selecting loci within the genome likely to have insertions and deletions and interpreting the results obtained. Although insertions, deletions, and single nucleotide polymorphisms (SNPs) produce detectable changes in melting curves (*32,33*), melting curves are qualitative rather than quantitative. In addition, melting curves alone cannot identify specific insertions, deletions, or quasispecies (SNPs) without the addition of probes for the affected target region of the genome or the use of PCR (*34,35*). Finally, because the strategy reported here tests for size polymorphisms, it does not require prior knowledge of the specific sequences that may have been introduced into (or deleted from) the pathogen genome to identify genetically modified organisms. However, this strategy does have 3 limitations.

First, sequences identical to (or cross-reactive with) host sequences cannot be used as targets because blood and tissue specimens are inevitably contaminated with host DNA (this issue can be resolved by searching the GenBank database). Second, the threshold of detection for genetically modified organisms is the addition (or removal) of sequences ≥5 bp (based on the sensitivity of capillary electrophoresis), i.e., point mutations (SNPs = quasispecies) (*36–38*) cannot be detected with this strategy. As a result, this method is likely to be of greater value for organisms with dsDNA genomes such as bacteria, eukaryotic parasites, and dsDNA viruses (in which quasispecies are less common because of more accurate replication) than for organisms with single negative-stranded RNA genomes (in which quasispecies are more common because their replication depends on the error-prone reverse transcriptase—HIV, hepatitis C, hepatitis B) (*39,40*). Third, capillary electrophoresis may need to be performed separately for each allotype to avoid confusion between amplicons of similar size from different allotypes (Figures 1–3).

Conclusions

The strategy reported here can be used for epidemiolog-

Table 4. Evidence for insertions, deletions, and repeats in human pathogens*

Pathogen	Genomic site of variation	Observed size variations	Reference
Plasmodium falciparum	Block 2 variable region of merozoite surface protein 1 (*msp1*), PCR	150–200 bp with multiple 9-bp insertions and deletions based on number of tripeptide repeats	(*14*)
Dengue	3′ NCR after the NS5 stop codon	2–14 and 75-nt deletions, 4 copies of 8-nt imperfect repeat	(*16*)
Yellow fever virus	3′ NCR	216-nt duplication, 40-nt deletion (repeat hairpin motif)	(*17*)
HIV	*env* gene	35- and 48-nt insertions, 21- and 36-nt deletions	(*18*)
	gp120 V3 and V4 loops	9- and 12-nt deletions	(*19*)
Mycobacterium tuberculosis	Novel *IS*6110 insertions†	36-bp DRs interspersed with variable spacers for DVRs	(*20*)
	VNTRs	Repeating units of 53–79 bp with 16–17 copies	(*21*)
	Genomic deletions	Based on genomic microarrays	(*21*)
Bacillus anthracis	MLVA†	Variations of 12, 9, 18, 72, and 5 bp for MLVA markers *vrrA*, *vrrB1*, *vrrB2*, *vrrC2*, and CG3	(*23*)
	Subtyping of 2001 bioterrorism organism	All isolates were genotype 62	(*24*)
Yersinia pestis	MLVA† with 25 markers for tandem repeat loci with 9–60 bp repeats of 3–36 units	Amplicon sizes for complete alleles ranging from 119 to 786 bp	(*25*)
Smallpox virus	Coding regions of the viral genome	Variable numbers of 9- and 21-bp repeats (n = 5–31 and 15–38, respectively), insertions of 32 and 464 bp and a 251-bp deletion	(*26*)
	Inverted terminal repeats between nonrepetitive elements 1 and 2 (NR1, NR2)	0–4 copies of a 69-bp sequence	
	Potential virulence proteins	Smallpox inhibitor of complement enzymes, chemokine-binding protein II, and Z-DNA binding protein	(*27*)

*PCR, polymerase chain reaction; NCR, noncoding region; DRs, direct repeats; DVRs, direct variant repeats; VNTRs, variable numbers tandem repeats; MLVA, multiple locus VNTR analysis; msp1, merozoite surface protein 1.
†Exists at multiple sites within the pathogen genome.

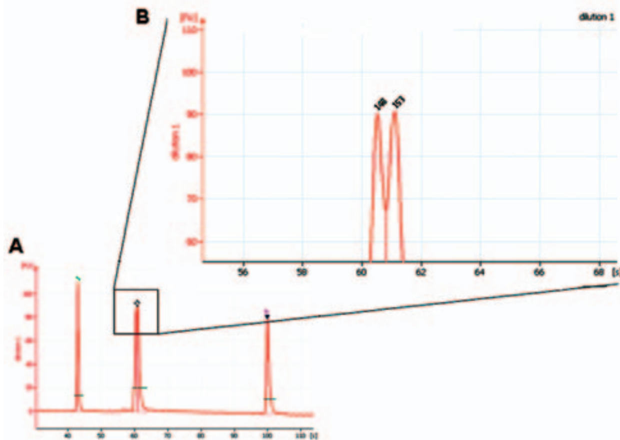

Figure 3. Capillary electrophoresis separation of polymerase chain reaction amplicons differing by 5 bp.

ic studies of simultaneous infection with multiple pathogens (genotypes) of the same species in emerging infectious diseases and for the rapid identification of select agents that have been genetically modified to increase their virulence or antimicrobial drug resistance.

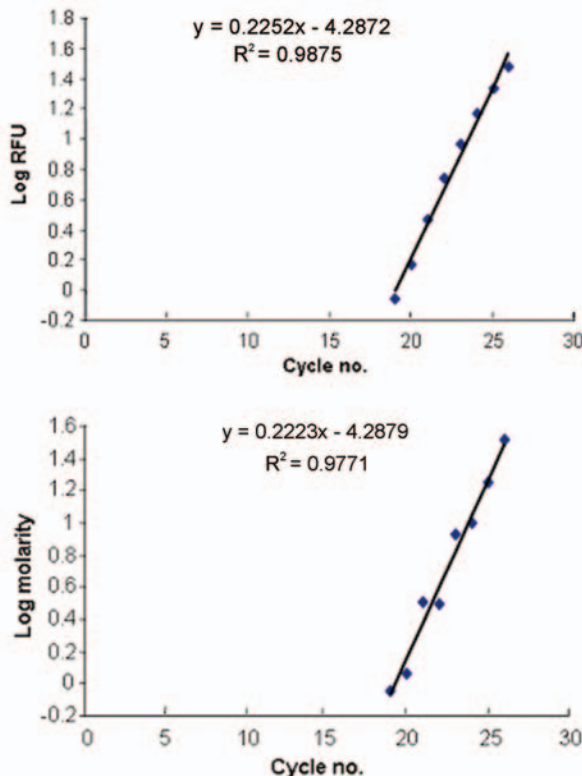

Figure 4. Comparison of amplicon concentration based on relative fluorescence from real-time polymerase chain reaction with peak area from capillary electrophoresis.

Acknowledgments

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"*Candidatus Rickettsia kellyi*," India

Jean-Marc Rolain,* Elizabeth Mathai,† Hubert Lepidi,* Hosaagrahara R. Somashekar,† Leni G. Mathew,† John A.J. Prakash,† and Didier Raoult*

We report the first laboratory-confirmed human infection due to a new rickettsial genotype in India, "*Candidatus Rickettsia kellyi*," in a 1-year-old boy with fever and maculopapular rash. The diagnosis was made by serologic testing, polymerase chain reaction detection, and immunohistochemical testing of the organism from a skin biopsy specimen.

Human rickettsioses are infections of emerging importance in India, where increasing numbers of cases among residents and travelers have been reported recently (*1,2*). Nevertheless, these diseases are not well described in the literature and, to date, only serologic evidence of rickettsial infections has been reported, including murine typhus, scrub typhus, and unidentified spotted fever group (SFG) rickettsiosis (*1,3,4*). Moreover, the results of serologic testing are presumptive and should be interpreted with caution. SFG rickettsiosis is seldom diagnosed in India, probably because of a low index of suspicion and a relative lack of diagnostic facilities. Specific diagnostic methods are needed to identify unexpected SFG agents either by polymerase chain reaction (PCR) or by culture (*5*).

Few reports of rickettsioses in children from southern India have been reported. Here we report the case of a 1-year-old boy with a new SFG rickettsiosis characterized by a maculopapular rash on the palms and soles. The diagnosis was confirmed by serologic testing, molecular detection, and immunohistochemical testing of a skin biopsy specimen. We propose the name "*Candidatus Rickettsia kellyi*" in honor of Professor Patrick Kelly, who has greatly contributed to the current knowledge of rickettsiae throughout the world.

The Study

A 1-year-old boy from Thiruppathur, Tamil Nadu, India, was brought for treatment; he had exhibited fever for 10 days and a maculopapular rash on the face and chest that had spread rapidly to the trunk and limbs. The rash was also on his palms and soles. No tick bite was noted. A skin biopsy was taken from a maculopapular lesion. Laboratory tests showed a leukocyte count of 15,300/mm^3, hemoglobin level of 9.2 g/dL, and normal platelet count, and normal cerebrospinal fluid was seen by lumbar puncture. The patient was given doxycycline syrup and cefotaxime because the diagnosis was not definitive and the boy was very ill. The boy responded dramatically and eventually recovered. Results of conventional culture of cerebrospinal fluid, skin biopsy specimens, and blood culture were negative.

With Weil-Felix agglutination assay, the serum sample taken at admission was weakly positive with OX-2 antigen (titer 40) and negative for OXK and OX-19 antigens, giving presumptive evidence of a rickettsial infection. DNA was extracted from the skin biopsy specimen and used as a template in 2 previously described PCR assays that targeted a portion of the rickettsial *ompA* gene as well as a portion of the rickettsial *gltA* gene, *ompB* gene, and *sca4* genes (*6,7*). Amplification products of the expected size were obtained from this extract but not from any concurrently processed control materials. The most closely related rickettsial species was found to be *R. honeï* with pairwise nucleotide sequence homologies of 92.3% for *ompA*, 99.2% for *gltA*, 94.6% for *ompB*, and 99.1% for *sca4*. Histopathologic testing of the skin biopsy specimen showed a leukoclastic vasculitis, and immunohistochemical testing by using a rabbit polyclonal antibody directed against SFG rickettsiae showed positive result (Figure 1).

For specific microimmunofluorescence assay, a panel of 13 rickettsial antigens, including SFG rickettsiae (*R. conorii* subsp. *indica*, *R. japonica*, *R. honei*, *R. helvetica*, *R. slovaca*, AT1 *Rickettsia*, *R. felis*, "*R. heilongjiangensis*"), typhus group rickettsiae (*R. typhi*), *Orientia tsutsugamushi* (strains Gilliam, Kato, Karp, and Kawazaki),

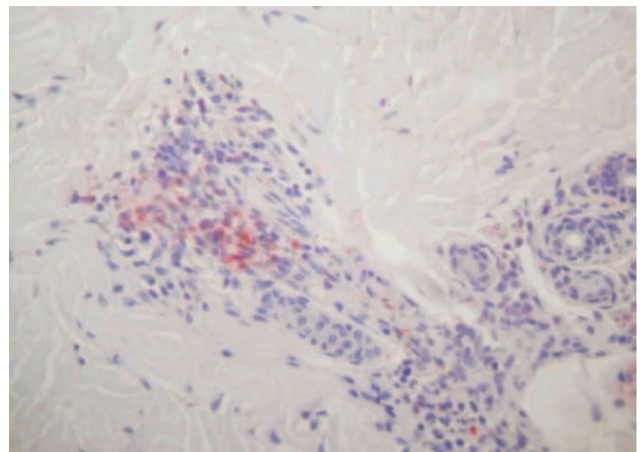

Figure 1. Immunohistochemical results showing rickettsiae in inflammatory infiltrates of the dermis (polyclonal rabbit anti-*Rickettsia* sp. antibody using a dilution of 1:1,000 and hematoxylin counterstain; original magnification ×250).

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Anaplasma phagocytophilum, *Ehrlichia chaffeensis*, and *Coxiella burnetii*, was used as previously described (*8,9*). The serum sample from the patient at the acute stage of illness was weakly positive for all SFG rickettsia with a negative immunoglobulin G (IgG) titer and a IgM titer from 64 to 128 according to the species tested. Serum was also positive by Western blot analysis as previously reported (*8*), but the species remained undetermined.

Conclusions

Clinical and laboratory data for this patient suggest that he had an SFG rickettsial infection that was confirmed by 3 different testing methods: serologic, immunohistochemical, and molecular based. Although no tick bite and no eschar were noted, the infection could have been acquired from any of a wide variety of arthropods in this area. In India, serologic evidence of human SFG rickettsioses has been found (*1,3,10*), but the epidemiology of etiologic agents is deduced only by serologic testing performed by using known rickettsial antigens. Our case, to the best of our knowledge, is the first human SFG rickettsiosis case diagnosed in India that was laboratory confirmed by using specific and direct detection of a rickettsial strain. Moreover, according to genetic guidelines for the classification of rickettsial isolates (*6*), our rickettsial strain found in the skin biopsy specimen belongs to a new species. Unfortunately, because the skin biopsy specimen was stored in alcohol, culture and complete phenotypic description of this isolate were not possible. The most closely-related rickettsial strain, according to genetic guidelines, was *R. honeï* as shown in the phylogenetic tree (Figure 2). *R. honeï* is the etiologic agent of Flinders Island (Australia) spotted fever, which was isolated from the blood of 2 patients in 1993 (*11,12*). The main reservoir of *R. honeï* was later determined to be *Aponomma hydrosauri*, a reptile tick (*13*). The pathogenicity of the original isolate of *R. honeï* (Thai tick typhus strain TT-118)

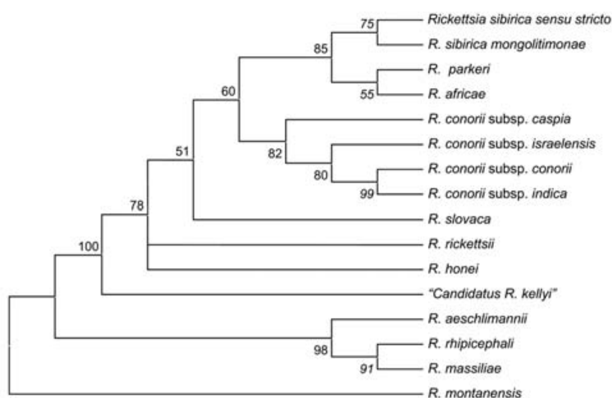

Figure 2. Phylogenetic tree of rickettsiae, including "*Candidatus Rickettsia kellyi*," obtained by comparison of partial sequences of *ompA* with the parsimony method.

for humans has not yet been confirmed, but it is possibly responsible for SFG human rickettsiosis in Thailand (*14*). Thus, genotyping of these strains is needed to better understand the epidemiology of SFG rickettsiosis in Asia. Further studies are needed to isolate and establish this new pathogenic SFG rickettsial strain from humans to confirm our case report. Moreover, tick species prevalent in this area of South Asia should be tested to find the rickettsial reservoir and increase understanding of the epidemiology of this rickettsial infection. New pathogens remain to be discovered in India, and new rickettsial diseases represent a challenge.

The partial ompA gene sequence of *"Candidatus Rickettsia kellyi"* has been deposited in the GenBank data library under accession no. DQ080005. GenBank accession nos. were TTU59726 for *gltA*, AF123724 for *ompB*, and AF163004 for *sca4*.

Dr Rolain is a researcher at the Unité des Rickettsies, the national reference center for rickettsiosis and WHO collaborative center in Marseille, France. The laboratory studies emerging and reemerging bacteria and arthropodborne diseases.

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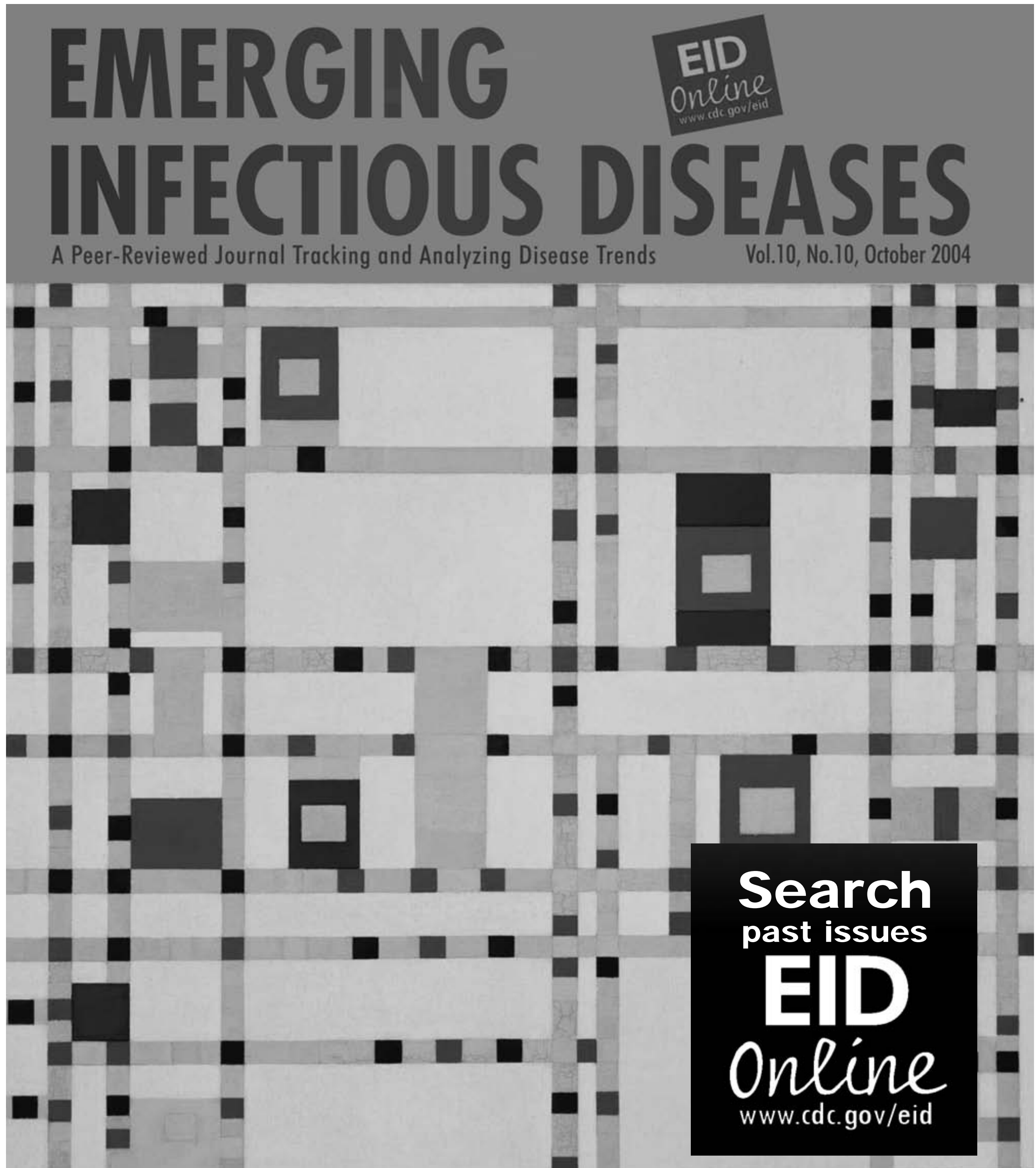

Lyssavirus Surveillance in Bats, Bangladesh

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Lyssavirus surveillance in bats was performed in Bangladesh during 2003 and 2004. No virus isolates were obtained. Three serum samples (all from *Pteropus giganteus*, n = 127) of 288 total serum samples, obtained from bats in 9 different taxa, neutralized lyssaviruses Aravan and Khujand. The infection occurs in bats in Bangladesh, but virus prevalence appears low.

Bats are known reservoirs of viruses in the *Lyssavirus* genus, family *Rhabdoviridae*. In the Americas, bats maintain the circulation of different lineages of rabies virus (RABV, genotype 1) (*1*). Lagos bat virus (genotype 2) and Duvenhage virus (DUVV, genotype 4) were isolated from bats in Africa (*2*). European bat lyssaviruses, types 1 and 2 (EBLV-1 and EBLV-2, genotypes 5 and 6, respectively) circulate in European bat populations (*3*). West Caucasian bat virus, a new putative lyssavirus genotype, was recently isolated from a bat in southern Europe (*4*). Australian bat lyssavirus (ABLV, genotype 7) was isolated from different genera of Australian bats (*5*).

Data on rabies in Asian bats are limited because of a lack of a suitable surveillance system. Only a few investigators reported presumable RABV isolates of bat origin in India and Thailand, but these were not corroborated, nor were reports about bat rabies in Siberia and Uzbekistan (*6*). No confirmed genotype 1 lyssavirus isolates are available from bats outside the Americas, to date. Recently, 3 lyssaviruses (Aravan, Khujand [*6*], and Irkut [*4*] viruses) were isolated from bats in different locations of Asia. These representatives were suggested as 3 new putative genotypes of the *Lyssavirus* genus, according to their genetic properties. Moreover, antibodies to lyssaviruses have been demonstrated in serum specimens of bats from the Philippines, Cambodia, and Thailand (*7–9*). In this article, we extend information on the geographic distribution of rabies among Asian bats and describe a limited survey in Bangladesh for evidence of lyssavirus activity.

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The Study

This project began as part of a larger study concerned with a suspected Nipah virus outbreak in the region. Active surveillance of bats was performed in 3 districts of Bangladesh: Meherpur and Naogaon during March 2003 and Rajbari during February and March 2004 (Figure 1). The animals were collected randomly from different roosts, trees, and fruit plantations.

Bats were anesthetized by a 0.05- to 0.1-mg intramuscular injection of ketamine hydrochloride. Injured bats and those that had clinical signs and symptoms were euthanized under sedation by exsanguination. Blood was transferred to serum separator tubes and was refrigerated until centrifugation. Serum was decanted into individual screw-topped vials. When possible, bats were identified to species. Brains of all euthanized bats were removed at necropsy and placed into individual sterile containers. Additional organs and oral swabs were also obtained from each bat. All specimens were held in temporary storage at –18°C (after collection) and later placed at –80°C. Carcasses of representative specimens were placed in formalin for archival purposes.

Bat brains collected in 2003 (N = 212, Table) were tested by the direct fluorescent-antibody test (DFAT) (*10*), by using both monoclonal (Centocor Inc., Malvern, PA, USA) and polyclonal (Chemicon International, Temecula, CA, USA) fluorescein isothiocyanate–labeled anti–rabies virus antibodies, and were subsequently processed for isolation

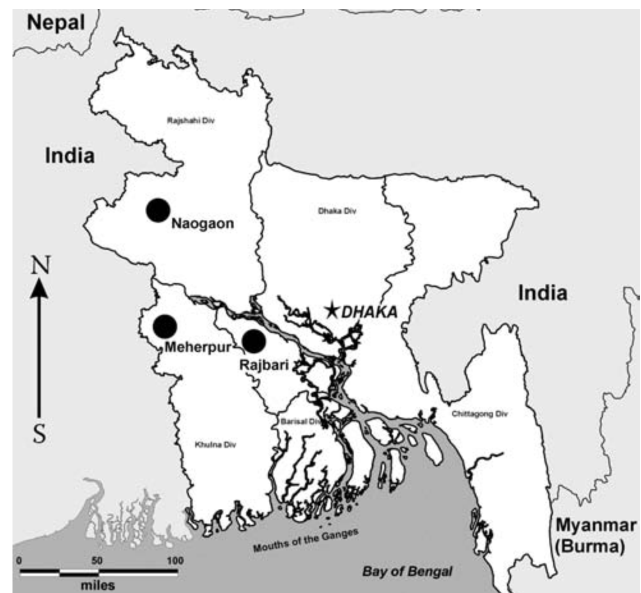

Figure 1. Map of Bangladesh with bat surveillance regions indicated (circles).

Table. Samples of bats captured in Bangladesh and subjected to lyssavirus diagnosis, isolation, and antibody detection*

	Brains		Sera	
Species	DFAT and MIT	DFAT only	1:20†	1:50
Pteropus giganteus	37	97	123	4
Cynopterus sphinx	6	5	6	1
Macroglossus sobrinus	0	1	1	0
Rousettus leshenaulti	0	46	11	0
Megaderma lyra	47	2	24	23
Pipistrellus sp.	85	0	24	37
Scotophilus heathii	30	0	15	12
S. kuhlii	1	0	1	0
Taphozous saccolaimus	6	0	2	4

*DFAT, direct fluorescent antibody test; MIT, mouse inoculation test.
†Starting dilutions available for rapid fluorescent focus inhibition test.

by the mouse inoculation test (MIT) (*11*). If death occurred during the MIT, mouse brains were subjected to the DFAT, and a second intracerebral passage was conducted with 5% brain suspensions, using 0.22-µm filters (Millipore Corp., Bedford, MA, USA). Bat brains collected in 2004 (N = 151, Table) were subjected to the DFAT only.

The presence of virus-neutralizing antibodies was determined by an adaptation of the rapid fluorescent focus inhibition test (RFFIT), as described (*7*). Because of volume limitations and the cytotoxicity of some specimens, 207 serum samples were available for testing at a starting dilution of 1:20, and 81 samples at a starting dilution of 1:50 (Table). Four different lyssaviruses were used in an initial in vitro screening: Aravan, Khujand, Irkut, and ABLV. When a positive result was obtained, testing on the sample was repeated, and comparative assays undertaken for additional lyssaviruses: EBLV-1, EBLV-2, DUVV, and RABV (e.g., routine rabies challenge virus standard, CVS-11). The dose of each virus used for the RFFIT was ≈50 infectious units per 100 µL. The duration of the test was 20 hours for ABLV and CVS-11 and 40 hours for other challenge viruses.

No evidence of lyssavirus antigen was detected in any bat brain by DFAT, and no neurotropic viruses were isolated by mouse inoculation. If a limited number of deaths occurred during the initial MIT (1 or 2 mice of 5 infected), those effects were not reproduced during the subpassage by filtration, which suggests that bacterial contamination of the field samples caused the death of mice during the initial MIT.

One serum sample repeatedly demonstrated neutralizing activity against Khujand virus, at a titer of 54. This sample was obtained from a young female giant Indian flying fox (*Pteropus giganteus*) (Figure 2) captured in the Meherpur District in 2003. Two other serum samples obtained from the same species (1 male and 1 female) in Rajbari District in 2004, neutralized Aravan and Khujand viruses at titers of 14–16. For these latter samples, neutralization was detected at dilutions of 1:20 and 1:25 but not detected at a dilution of 1:50. The titers of all serum specimens were <10 (i.e., no neutralization occurred in the dilution of 1:20) against other lyssaviruses.

Conclusions

We have presented serologic evidence of lyssavirus infection in bats from Bangladesh. Antigenic cross-reactivity has been reported among Aravan, Khujand, and other members of the *Lyssavirus* genus (*12*). Therefore, detectable antibody may cross-react with other related lyssaviruses, as well as with viruses yet to be discovered.

Based upon this preliminary survey, a low prevalence of lyssavirus infection appears in *P. giganteus* in Bangladesh. Thus, it is not surprising that all brain samples collected were negative for detection of lyssavirus antigen. Because different lyssaviruses and bat species are found in Asia, and therefore different virus-host interactions would be expected in the region, extrapolating antibody-positive/virus-positive ratios, as have been estimated from American (*13,14*) or European (*15*) bat populations, would be difficult.

Further surveillance for Asian bat lyssaviruses should be conducted. Public health authorities need to be aware of

Figure 2. Giant Indian flying foxes (*Pteropus giganteus*). (Photo by I.V. Kuzmin.)

the potential for bats to transmit lyssaviruses, and public education of this potential should be enhanced. Frugivorous bats forage in fruit plantations in many regions of the Old World tropics, including Bangladesh. Indigenous people capture and may consume these animals. Direct contact between humans and bats frequently occurs during these interactions. Absence of current information on human rabies after bat exposure may be a result of inadequate education, incomplete surveillance, and lack of characterization of viruses from rabies cases (*6*).

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Dr Kuzmin is an International Emerging Infectious Diseases fellow in the Rabies Section, Centers for Disease Control and Prevention. His professional interests include lyssavirus natural history, with particular attention to bats.

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Molecular Analysis of Fluoroquinolone-resistant *Salmonella* Paratyphi A Isolate, India

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Salmonella enterica serovar Paratyphi A is increasingly a cause of enteric fever. Sequence analysis of an Indian isolate showed a unique strain with high-level resistance to ciprofloxacin associated with double mutations in the DNA gyrase subunit *gyrA* (Ser83→Phe and Asp87→Gly) and a mutation in topoisomerase IV subunit parC (Ser80→Arg).

Salmonella enterica serovar Paratyphi A is the second most common cause of enteric fever after *S*. Typhi. Approximately 0.25 *S*. Paratyphi A infections (paratyphoid fever) occur for each *S*. Typhi infection (typhoid fever) (*1*). Given global estimates of >21 million cases of typhoid fever in the year 2000, >5 million cases per year of *S*. Paratyphi A probably occur. Paratyphoid fever is a major clinical problem in India, but large outbreaks were not reported until 1996 (*2*). Elsewhere, in southern China for example, extensive outbreaks are also occurring (*3*).

Since 1998, plasmid-mediated multidrug resistance in *S*. Paratyphi A, associated with chromosomally mediated reduced susceptibility to ciprofloxacin, has caused concern (*4*). Reduced susceptibility to fluoroquinolones results in a poor response of salmonellosis patients to treatment and may allow prolonged bacterial shedding (*5*). Rising resistance to fluoroquinolones is likely to be driving an increase in cases of paratyphoid fever in regions where fluoroquinolones are used empirically to treat enteric fever. We must monitor the emergence of resistance in this enteric pathogen to differentiate between the acquisition of resistance during treatment (mutations occurring in different bacterial strains) or clonal expansion of a successful strain by person-to-person spread (identical mutations associated with a single strain). To do so, we need to describe the molecular basis of resistance and the genotype of the resistant strains. High-level ciprofloxacin-resistant *S*. Paratyphi A (MIC 8 μg/mL) is present in India (*6*) and Japan (MIC ≥128 μg/mL) (*7*). However, because S. Typhi, the major cause of enteric fever in India, has not yet developed high-level resistance to fluoroquinolones, enteric fevers are often treated empirically with fluoroquinolones. If this trend continues, fluoroquinolone-resistant strains of *S*. Paratyphi A are almost certain to become a major cause of enteric fever in many areas.

We analyzed, by DNA sequencing, the DNA gyrase and topoisomerase IV genes of the first reported highly fluoroquinolone-resistant *S*. Paratyphi A isolate (*6*). We looked at the full coding sequence, including the quinolone resistance–determining region (QRDR), of both subunits of DNA gyrase and topoisomerase IV for mutations associated with resistance to fluoroquinolones. We also used multilocus sequence typing (MLST) to confirm the identity of the isolate.

The Study

The strain described here (Pond1) was first isolated in Pondicherry, India, in November 2002 from the blood of a 23-year-old man admitted with fever and with no history of having received antimicrobial chemotherapy (*6*). The isolate was resistant to ciprofloxacin and nalidixic acid, and the MIC of ciprofloxacin was 8 mg/L. It was sensitive to all other antimicrobial drugs tested by disk diffusion: ampicillin, chloramphenicol, cotrimoxazole, gentamicin, and ceftriaxone. Repeat testing showed that zones of inhibition indicating susceptibility were seen around both ofloxacin (17 mm) and ciprofloxacin (21 mm) 5-μg disks; however, in light of the ciprofloxacin MIC and resistance to nalidixic acid, Pond1 was considered resistant to fluoroquinolones. No similar isolates have been seen in this area since the initial report, although the total number of paratyphoid fever cases has increased.

Polymerase chain reaction (PCR) amplification (Table 1) and direct DNA sequencing of both strands of the full length of gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) subunit genes was performed with an ABI Prism dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA, USA) on an ABI 3730 automated sequencer. Results showed 3 mutations: 2 in *gyrA* and 1 in *parC*. A comparison of these mutations with those previously described in fluoroquinolone-resistant *S*. Paratyphi A is shown in Table 2. A rise of fluoroquinolone resistance over time is apparent, and although the point mutations do not fully explain the MIC data, we noted general associations: a single mutation in *gyrA* is always associated with resistance to nalidixic acid and reduced susceptibility to ciprofloxacin and ofloxacin, and a double mutation in *gyrA* is always accompanied by mutations in *parC* and is asso-

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Table 1. Primer sequences for amplification of topoisomerases

Primer	Sequence 5′–3′	Amplicon	Gene (coding sequence length)
gyrA7 (F)	5′ GGGTCGACTGATTATGGTTTATGCCTCC 3′	3199	2637
gyrA25 (R)	5′ GAGACTTTCAGCGTAGTTCG 3′		
gyrB1 (F)	5′ TTGCCTCTGAACTTGACGATGC 3′	2762	2415
gyrB9 (R)	5′ GAAGTCGCTGACCTGCTCAC 3′		
parC3 (F)	5′ CGATTTTCCGGTCTTCTTCCAG 3′	2616	2259
parC10 (R)	5′ GCAATGCACGAATAAACAACGG 3′		
parE3 (F)	5′ CCTGATCTGGCTACTGCAACAG 3′	2183	1893
parE8 (R)	5′ ATGCGCAAGTGTCGCCATCAG 3′		

ciated with high-level (>4 μg/mL) resistance. Although this sample is limited, heterogeneity in mutations found at all sites suggests that this finding is not the result of a single successful strain's sequentially acquiring mutations but rather that resistance is arising in several different strains.

Conclusions

The QRDR within topoisomerases contains hotspots for mutations around the active site, which are associated with raised MIC values for fluoroquinolones (*10*). For *gyrA* from nalidixic acid–resistant *Salmonella* isolates, 2 mutations are most frequently observed in clinical isolates: Ser83→Phe and Ser83→Tyr (*8,9,11*). The association with resistance of mutations seen in *parC*, however, is less clear (*12*). Mutations in *parC* of gram-negative bacteria are usually within the QRDR at amino acids 80 and 84 (Ser 80→Ile, Glu 84→Gly, Lys). The first reported mutation was Thr57→Ser, and other mutations have been described: Asp 79→Ala, Gly 78→Asp (*9*). Each mutation, in *gyrA* or *parC*, can give rise to different MICs in different isolates, which means that other factors must also influence the resistance phenotype in *S.* Paratyphi A. The most likely cause is changes in expression levels of proteins involved with permeability barriers and efflux pumps. These changes could be the result of either point mutations in transcription promoters and regulators or downstream effects of mutations in topoisomerases. Known mechanisms of fluoroquinolone resistance that we were able to screen for include transmissible plasmidborne resistance and efflux pumps. The *qnr*-containing plasmid was not detected with PCR using primers 5′ GGG TAT GGA TAT TAT TGA TAA 3′ and 5′ CTA ATC CGG CAG CAC TAT TA 3′ (*13*) in Pond1, and sensitivity testing gave the same zone size around both tetracycline and chloramphenicol discs when compared with a nalidixic acid–susceptible isolate. This finding, combined with the ciprofloxacin MIC of 8 μg/mL, argues against the presence of multiple antimicrobial drug resistance efflux pumps. Thus the high-level resistance seen in Pond1 appears to be associated directly with 3 point mutations in the topoisomerase genes.

To confirm the identity of the isolate as *S.* Paratyphi A, we used MLST. The primer sequences and MLST data are available from the MLST database at the Max-Planck-Institut für Infektionsbiologie (http://web.mpib-berlin.mpg.de/mlst/). Six of 7 sequenced loci matched exactly the previously described sequences, and 1 was a unique allele. This finding means that the isolate described here is within the clonal MLST group described as *S.* Paratyphi A but is a recognizable variant. This finding supports previous typing data that show very little variation (*14*); typing *S.* Paratyphi A is problematic because genomic restriction analyses (pulsed-field gel electrophoresis) of isolates from an outbreak are not always identical, and susceptible and resistant strains cannot be differentiated. For molecular epidemiologic studies to be carried out, several methods need to be used (*15*). A broader study of single base-pair differences between strains of *S.* Paratyphi A could provide a usable typing scheme.

Table 2. Presence of *qnr* and mutations in DNA gyrase and topoisomerase IV genes of *Salmonella enterica* serovar Paratyphi A strains associated with decreased susceptibility or resistance to ciprofloxacin*

Country	Year	MIC (μg/mL)		*qnr*	*gyrA*	*parC*	Reference
		Cp	Nal				
India	1999	0.38	>256	ND	Ser83→Phe	ND	(*8*)
Bangladesh	1999	0.5	>256	ND	Asp87→Gly	ND	(*8*)
India	1999	0.5	>256	ND	Ser83→Phe	ND	(*8*)
Hong Kong	2000	0.5	>256	ND	Ser83→Tyr	NM	(*9*)
Japan	2002	≥128	>256	ND	Ser83→Phe, Asp87→Asn	Glu84→Lys	(*7*)
India	2002	8	>256	NP	Ser83→Phe, Asp87→Gly	Ser80→Arg†	This study

*No mutations were detected in *gyrB* and *parE*. Cp, ciprofloxacin; Nal, nalidixic acid; ND, not determined; NP, not present; NM, no mutation within the quinolone resistance–determining region.
†European Molecular Biology Laboratory accession no. AM050347.

Resistance in *S.* Paratyphi A populations must be monitored because the acquisition of resistance to fluoroquinolones, coupled with the reduction in *S.* Typhi by the use of typhoid-specific vaccination, may cause *S.* Paratyphi A to become the main cause of enteric fever. Disc susceptibility testing does not always detect resistance, and screening with nalidixic acid and MIC testing remains the method of choice. The isolate described here, Pond1, contains a unique combination of mutations that provides a way to track the spread of this strain of *S.* Paratyphi A.

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Dr Nair is a research associate in tropical bacteriology at The Wellcome Trust Sanger Institute, The Wellcome Trust Genome Campus, United Kingdom. His research includes the use of molecular tools to detect genomic diversity of drug-resistant/drug-susceptible *Salmonella* spp. from different geographic and epidemiologic backgrounds and effects of drug resistance on the biology of the organism.

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Canine Coronavirus Highly Pathogenic for Dogs

Canio Buonavoglia,* Nicola Decaro,* Vito Martella,* Gabriella Elia,* Marco Campolo,* Costantina Desario,* Massimo Castagnaro,† and Maria Tempesta*

Canine coronavirus (CCoV) is usually responsible for mild, self-limiting infections restricted to the enteric tract. We report an outbreak of fatal disease in puppies caused by a pathogenic variant of CCoV that was isolated from organs with severe lesions.

Coronaviruses are large, enveloped, positive-stranded RNA viruses (*1*). Three different coronaviruses have been identified in dogs (*2,3*). Canine coronavirus (CCoV) type I and type II are included in group 1 coronaviruses, and their evolution is related to that of feline coronavirus (FCoV) type I and type II. FCoV type II originated by heterologous recombination between CCoV type II and FCoV type I, while CCoV type I is genetically more similar to FCoV type I than to CCoV type II (*3*). In addition, 2 FCoV biotypes that differ in pathogenicity have been observed in cats.

The onset of acute fatal disease (feline infectious peritonitis) is caused by pantropic variants (able to disseminate throughout the organism) of enteric FCoVs with deletions or recombinations in the 3c and 7b genes at the 3′ end of the FCoV genome (*4*). Similarly, changes in tissue tropisms in porcine and murine coronaviruses (*5,6*) and adaptation of the recently recognized severe acute respiratory syndrome–associated coronavirus (*7*) to humans have been related to mutations or deletions. A third canine coronavirus, CRCoV, detected in the respiratory tract, has ≤96.0% amino acid (aa) conservation in the spike (S) protein with bovine coronavirus within group 2 coronaviruses, which provides strong evidence for a recent host-species shift (*2*).

Coronavirus infection in dogs is usually restricted to the enteric tract. The infection is self-limiting and in general produces only mild or asymptomatic forms of enteritis (*8*). We report the identification of a pantropic, highly pathogenic variant of CCoV type II.

The Study

In May 2005, a severe outbreak of fatal systemic disease occurred in a pet shop in Bari, Italy. Clinical symptoms were initially observed in 3 miniature pinschers (45 days of age) and 1 cocker spaniel (53 days of age) and consisted of fever (39.5°C–40°C), lethargy, inappetence, vomiting, hemorrhagic diarrhea, and neurologic signs (ataxia, seizures) with death after 2 days. The same symptoms were observed 3–4 days later in 2 other miniature pinschers (45 days of age) and 1 Pekinese (56 days of age). Necropsy of the dogs showed hemorrhagic enteritis, abundant serosanguineous fluid in the abdominal cavity, and severe lesions in the parenchymatous organs. The lungs had multiple, patchy, red areas of consolidation. Livers were yellow-brown and congested, with hemorrhages on their surfaces, and spleens were enlarged with subcapsular hemorrhages. Variable gross changes in other organs included multifocal hemorrhagic renal cortical infarcts and petechial hemorrhages on lymph node surfaces.

Virologic and bacteriologic investigations on the parenchymatous organs did not detect common canine pathogens, notably canine parvovirus type 2, canine distemper virus, canine adenovirus type 1 and type 2. CCoV type I and type II were identified in the intestinal contents of all puppies by genotype-specific real-time reverse transcription–polymerase chain reaction (RT-PCR) assays (*9*). CCoV type II RNA was also detected in lungs (median 1.08×10^6 RNA copies/µL of template), spleen (median 4.46×10^6 RNA copies/µL of template), liver (median 9.02×10^4 RNA copies/µL of template), kidney (median 7.54×10^4 RNA copies/µL of template), and brain (median 5.23×10^3 RNA copies/µL of template). Virus-induced cytopathic effect was observed in A-72 cells, and CCoV type II strain (CB/05) was isolated from all tissues examined except brain tissue. Immunohistochemical analysis with a CCoV-specific monoclonal antibody detected CCoV antigen in the organs with gross lesions that were examined (lungs, kidneys, liver, spleen, gut, and lymph nodes) (Figure 1).

The sequence of the 3′ end of the genome (8.8 kb) of the pantropic CCoV strain was determined by RT-PCR amplification and sequencing of overlapping fragments. The S, envelope, and membrane proteins and nucleoprotein showed a high degree of amino acid identity with the cognate open reading frame (ORF) of CCoV type II. The S protein of strain CB/05 had the highest identity to FCoV type II strain 79-1683 (Figure 2). Comparison with strain CB/05 was possible only with CCoV type II strains Insavc-1 (*10*) and BGF (*11*) and CCoV type I strains Elmo/02 and 23/03 (*3,12*) because of a lack of data on the 3′ end of the CCoV genome in the genes encoding for nonstructural proteins (NSPs) 3a, 3b, 3c, 7a, and 7b. NSPs 3a, 7a, and 7b were not altered. NSP 3b (22 aa) was 49 aa shorter than expected because of a 38-nucleotide deletion and a frame shift mutation in the downstream sequence that introduced

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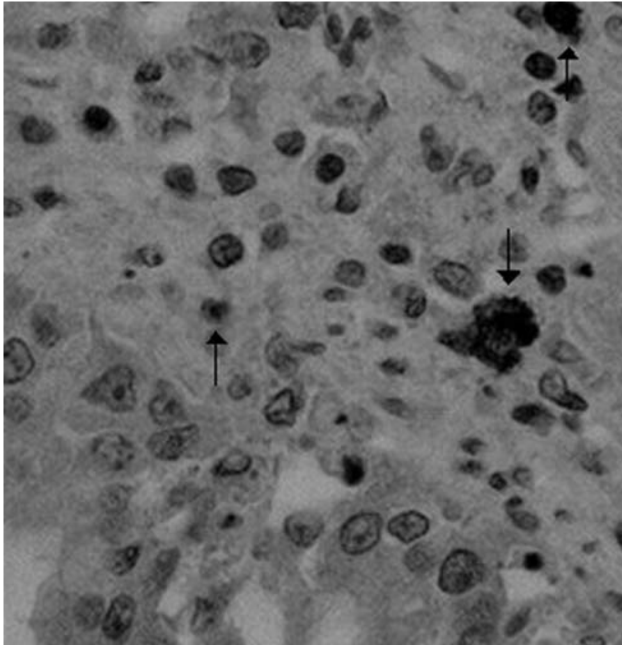

Figure 1. Immunohistochemical detection of canine coronavirus antigen (arrows) in canine lung tissue by a specific monoclonal antibody (magnification ×400).

an early stop codon. NSP 3c (244 aa) was 6 aa shorter and 79 aa longer than the cognate proteins of the enteropathogen strain BGF and the attenuated strain Insavc-1a, respectively.

To confirm the pathogenic potential of strain CB/05, we experimentally infected two 6-month-old dogs (authorization no. 67/2002-C released by Ministry of Health of Italy). Two milliliters of cryolysate of a lung-derived first-passage virus in A-72 cells were administered intranasally to the dogs. The cell cryolysate tested negative for other common canine pathogens and had a 50% tissue culture infectious dose of $10^{5.50}$/50 μL on A-72 cells and 1.18×10^7 RNA copies/μL of template by real-time RT-PCR. The virus was reisolated from the experimentally infected dogs. Severe clinical symptoms characterized by pyrexia (temperature 39.8°C–40.1°C), anorexia, depression, vomiting, diarrhea, and leukopenia were observed that persisted 8–10 days. Despite the severe symptoms, the dogs slowly recovered from their illness.

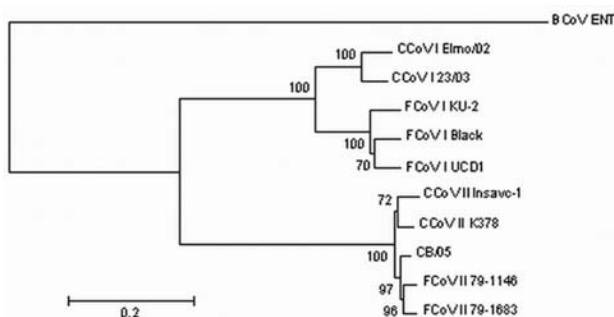

Figure 2. Neighbor-joining tree of the spike protein of canine coronavirus (CCoV) and feline coronavirus (FCoV). The following reference strains were used for phylogenetic analysis: CCoV type I strains Elmo/02 (GenBank accession no. AY307020) and 23/03 (AY307021); CCoV type II strains Insavc-1 (D13096) and K378 (X77047); FCoV type I strains KU-2 (D32044), Black (AB088223) and UCD-1 (AB088222); FCoV type II strains 79-1146 (X06170) and 79-1683 (X80799); and bovine coronavirus (BCoV) strain ENT (NC_003045). The tree is rooted on BCoV-ENT and drawn to scale. A statistical support was provided by bootstrapping >100 replicates. The scale bar represents 20 substitutions per 100 sequence positions.

Conclusions

Point mutations or deletions in the S protein and NSPs have been associated with changes in tropism and virulence of coronaviruses (*4–7,13*). CCoV strain CB/05 showed intact structural and nonstructural proteins, with an S protein closely related to that of other type II CCoVs. The only striking change was the truncated form of NSP 3b. Whether the deletion in the ORF of NSP 3b is involved in pathobiologic changes should be assessed with reverse genetic systems.

The present study describes for the first time the occurrence of fatal infections in dogs by coronaviruses. Experimental infection of dogs with the virus isolate resulted in a severe systemic disease that mimicked the clinical symptoms observed in the outbreak. However, the different ages at infection (6 months vs. <2 months) likely resulted in the disease being nonfatal. Accordingly, the appearance of pathogenic CCoV variants should always be regarded as a potential threat to domestic dogs and considered when unexplainable fatal disease outbreaks occur in puppies.

Epidemiologic studies are required to determine whether the pantropic CCoV strain is a new coronavirus variant emerging in canine populations or a widespread infectious agent of dogs that usually goes undetected. Vaccination trials could also help determine whether the CCoV vaccines currently available are effective against the highly virulent CCoV strain.

The 2002–2003 SARS epidemic has demonstrated that the study of animal coronaviruses is paramount to understanding the ecology and evolution of human coronaviruses. The coronaviruses of carnivores provide a paradigmatic model of how coronaviruses cross the species barriers, adapt to new host species, and change their pathogenicity.

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Dr Buonavoglia is professor of veterinary medicine at the University of Bari. His research interests include the study of viral pathogens of dogs, with particular emphasis on canine coronavirus.

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Acute Hemorrhagic Conjunctivitis and Coxsackievirus A24v, Rio de Janeiro, Brazil, 2004

Fernando N. Tavares,* Eliane V. Costa,* Silas S. Oliveira,* Cecilia C.A. Nicolai,† Meri Baran,† and Edson E. da Silva*

An outbreak of acute hemorrhagic conjunctivitis (AHC) occurred in Rio de Janeiro in 2004. Coxsackievirus A24v (CA24v) was identified as the etiologic agent, and partial sequences from the VP1 gene show that the isolates are closely related to CA24v viruses that previously caused AHC epidemics in South Korea and French Guiana.

Acute hemorrhagic conjunctivitis (AHC) is a rapidly progressive and highly contagious viral disease that is primarily caused by 2 distinct enteroviruses: enterovirus 70 (EV70) and a variant of coxsackievirus A24 (CA24v). These viruses have caused epidemics of AHC in tropical coastal regions throughout the world (*1*). The disease was first reported in Ghana, Africa, in 1969 and subsequently spread to several other countries of the Middle East, Asia, and Oceania (*1–3*). AHC caused by CA24v was first reported in 1970 during an epidemic in Singapore with 60,000 reported cases (*1*). Since then, outbreaks of AHC caused by CA24v have been regularly reported in several other countries (*1,4–9*), which highlights the high transmissibility of this agent. Ocular tropism is not limited to these serotypes, and other enteroviruses, e.g., echovirus 7 and 11, coxsackievirus B1 and B2, and non-enteroviruses (adenoviruses), can also cause conjunctivitis (*1*). The disease is characterized by sudden onset of ocular pain, swelling of the eyelids, a foreign body sensation or irritation, epiphora (excessive tearing), eye discharge, and photophobia. A palpebral conjunctival follicular reaction, subconjunctival hemorrhage, and congestion are common. Symptoms start after an incubation period of 12 to 48 hours, and the clinical signs usually disappear in 1 to 2 weeks (*1,3,8*).

We describe a CA24-related outbreak of AHC in Rio de Janeiro, Brazil, during April and May of 2004. The first clinical manifestations of the outbreak began in April of 2004 and were mainly restricted to the city of Rio de Janeiro. During the outbreak, >60,000 cases were officially reported to the state public health authorities. However, the actual number of cases was certainly much higher, since most patients seek medical assistance only during the beginning of an outbreak. The most frequent clinical manifestations of infections were as classically described: patients had sudden onset of ocular pain, foreign body sensation, irritation, epiphora, photophobia, and subconjunctival hemorrhage.

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The Study

Sterile cotton swabs were used to collect tears and eye discharges from 15 AHC patients. Each swab was collected in 1 mL viral transport medium (1× Hank's balanced salt solution containing 5% fetal bovine serum, penicillin [100 U/mL], and streptomycin [100 μg/mL], pH 7.4) in 5-mL cryovials. All clinical samples were kept on dried ice or at –70°C and transported to the enterovirus laboratory at Fiocruz, Rio de Janeiro.

Confluent HEp-2c and RD cells cultured in minimum essential medium supplemented with 2% of fetal bovine serum and antimicrobial drugs were used for viral isolation. Cell cultures were spread with 0.2 mL viral transport medium, incubated at 37°C, and observed for 7 days for cytopathic effect (CPE). One additional blind passage was performed if CPE was not observed during the first passage. Only HEp2c cells were capable of viral isolation, with characteristic CPE observed in 9 (53%) of 15 specimens.

RNA was extracted from 250 μL virus-infected culture supernatant by using Trizol LS (Invitrogen, Carlsbad, CA, USA), and the complementary DNA was synthesized with Oligo(dT)15 (Invitrogen) by using SuperScriptII reverse transcriptase (Invitrogen). Enterovirus group–specific reverse transcription–polymerase chain reaction (RT-PCR) was performed by using a primer pair (222/292) that amplifies an ≈350-bp fragment within the VP1 gene, as described (*10*). RT-PCR products were analyzed by electrophoresis in 1% agarose gels containing 0.5 μg/mL ethidium bromide. Products were further gel purified by using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and quantified by comparison, in 1% agarose gel, with Low DNA Mass Ladder (Invitrogen). Cycle-sequencing reactions were performed by using the ABI BigDye Terminator Cycle Sequencing Ready Reaction (PE Applied-Biosystems, Foster City, CA, USA) in a GeneAmp thermocycler. VP1 sequences from our isolates were compared to those available at GenBank by the BLAST software (*11*) to determine viral identity and serotype and aligned with sequences obtained from the database by using ClustalW (*12*). To identify respective divergence and infer the genetic relationship among the isolates, we used the neighbor-joining reconstruction method included in the Mega 3 software (*13*).

All isolates were identified as coxsackievirus A24v. The nucleotide sequence identities among CA24v isolates of the 2004 outbreak in Rio de Janeiro and CA24v isolates from a preceding AHC epidemic in Brazil in 2003 varied from 98.5% to 100%. The fact that several isolates, recovered 1 year apart from different AHC episodes, shared 100% identity could be partially explained by the lower resolution of the sequence interval used for the analysis (270 bp or ≈3.6% of the genome) when compared with the entire VP1 (≈900 nt or ≈12% of the enterovirus genomes), which is regularly used for poliovirus and enterovirus phylogenetic analysis. The sequence window can be widened to cover the complete VP1 or the complete genome if higher resolution is needed. Phylogenetic analyses based on the small genome segment generated by primers 222/292 provided sufficient information to identify the relationship among the CA24v isolates and the probable origin of AHC strains in circulation in Brazil. Both 2003 and 2004 isolates were closely related to the CA24v isolates (97.5%–99.0%) that caused AHC epidemics in South Korea and French Guiana in 2002 and 2003 (Figure).

Conclusions

During the first half of 2003, CA24v was also the etiologic agent responsible for a large outbreak of AHC in several states of Brazil, including Rio de Janeiro (unpub. data). The present outbreak (2004) also occurred during the end of summer and beginning of fall.

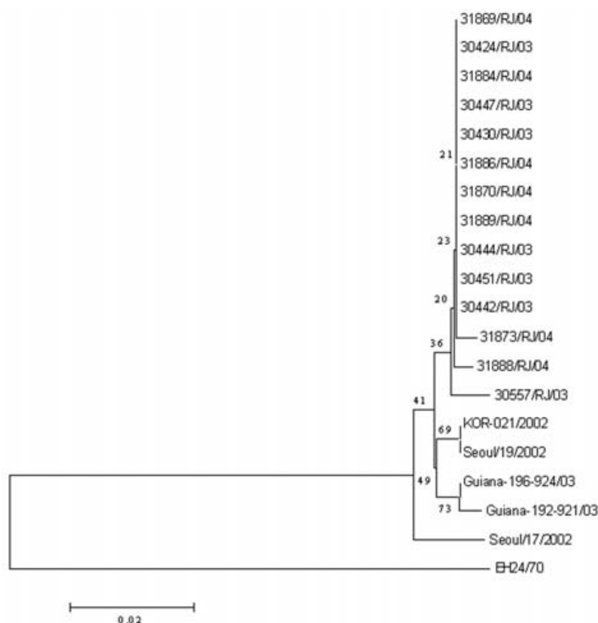

Figure. Phylogenetic analysis of CA24 strains isolated during the acute hemorrhagic conjunctivitis outbreaks in Rio de Janeiro in 2003 and 2004. Sequences of CA24 isolated from previous outbreaks in Korea in 2002 (Seoul/17/2002, Seoul/19/2002, and KOR-021/2002, GenBank accession nos. AY296249, AY296251, and AF545847, respectively), in French Guiana in 2003 (192-921-2003 and 196-924-2003, accession nos. AY876178 and AY876181, respectively), and in Singapore in 1970 (EH24/70, accession no. D90457) are included for comparison.

Sequencing analysis of a relatively small fragment in the VP1 is a simple and rapid method for enterovirus typing (*10*). Furthermore, comparisons of these sequences were useful to infer phylogenetic relationships among CA24v isolates. The CA24v responsible for the 2004 outbreak in Rio de Janeiro had >97% identity with the CA24v isolated in Korea and French Guiana from AHC outbreaks in 2002 and 2003, which suggests a direct route of disease dissemination among these countries. However, more data are required to support these findings. The source of the outbreak and factors that influenced its spread remain unclear.

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Dr Tavares completed this work as part of his master's thesis. He is currently affiliated with the enterovirus laboratory at Instituto Oswaldo Cruz, Brazil, and his research activities focus on the phylogenetic analysis of enteroviruses.

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Pneumonia and New Methicillin-resistant *Staphylococcus aureus* Clone

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Necrotizing pneumonia caused by *Staphylococcus aureus* strains carrying the Panton-Valentin leukocidin gene is a newly described disease entity. We report a new fatal case of necrotizing pneumonia. An *S. aureus* strain with an *agr*1 allele and of a new sequence type 377 was recovered, representing a new, emerging, community-acquired methicillin-resistant clone.

Necrotizing pneumonia associated with Panton-Valentine leukocidin (PVL) is a recent described clinical entity. It mainly occurs in children and young adults (median age 15 years), is fatal in 75% of cases, and is associated with a median survival time of 4 days (*1*). Necrotizing pneumonia is often preceded by a viral-like illness with signs of rhinopharyngitis. Viruses such as the influenza A virus have been isolated concomitantly with the PVL-positive *S. aureus* (*1*). Clinically, necrotizing pneumonia is mainly characterized by a rapidly extensive pneumonia often associated with pleural effusion and progressing towards the acute respiratory distress syndrome. Major associated signs include hemoptysis and leukopenia ($<2 \times 10^9$/L) (*1*). The severity of necrotizing pneumonia could be due to the products of the PVL genes, which are present in all *S. aureus* strains associated with this disease. The precise pathophysiology of the disease is unknown, but PVL causes tissue necrosis and leukocyte destruction. This toxin has been linked to primary skin and soft-tissue infections such as furuncles and abscesses (*1*).

Necrotizing pneumonia is mainly associated with PVL-positive methicillin-susceptible *Staphylococcus aureus* strains. Only 1 of the 16 cases reported by Gillet et al. (*1*) and 6 of the 40 cases of necrotizing pneumonia reported in the literature were associated with methicillin-resistant strains (MRSA). These PVL-positive MRSA correspond to clones that have emerged worldwide and cause community-acquired (CA) infections in patients with no risk factors. These CA-MRSA clones have a continent-specific distribution or worldwide distribution described by Vandenesch et al. (*2*). CA-MRSA clones that have spread worldwide share SCC*mec* type IV, the smallest cassette containing the *mecA* gene coding for methicillin resistance. The major European clone has a sequence type (ST) 80, as determined by multilocus sequence typing, and an *agr* (for accessory gene regulator) allele of type 3. *agr* is a global regulator controlling the expression of virulence factors in *S. aureus,* and has 4 alleles (*3*). PVL-positive CA-MRSA isolated in Australia have an *agr* type 3 allele and are of ST30 or ST93. The PVL-positive CA-MRSA isolated in the United States are ST1 and *agr* allele type 3, but some are ST8 or ST59 and *agr* allele type 1 (*2*). Here we report a fatal case of necrotizing pneumonia involving a 59-year-old woman infected by a new clone of PVL-positive CA-MRSA.

Case Report

A 59-year-old woman with an unremarkable medical history was admitted to Limoges University Hospital (France) in December 2003 for acute respiratory failure. She had a 3-day history of flulike syndrome with fever, chills, cough, and myalgia and had been prescribed nonsteroidal antiinflammatory therapy. On admission, she had dyspnea, nonproductive cough, and rales in both lungs. Her arterial pressure was 60/30 mm Hg. Skin rash and diarrhea were absent. A chest radiograph showed diffuse alveolar infiltration of both lungs, without pleural effusion. Laboratory studies showed leukopenia (leukocytes = 1.66×10^9/L), mild thrombocytopenia (115,000 platelets/mm^3), hypoxemia ($PaO_2/FiO_2 = 61$), and lactic acidosis (pH = 7.29, lactates = 4.45 mmol/L). Empirical antibacterial chemotherapy combining ceftriaxone, ciprofloxacin, and gentamicin was started on admission, and mechanical ventilation was immediately necessary. Repeated tracheal aspiration was highly productive and showed hemoptysis and sustained intraalveolar plasma leakage. Despite fluid resuscitation, continuous catecholamine perfusion, drotrecogin alfa (activated) administration (Xigris, Eli Lilly and Company, Indianapolis, IN, USA), and aggressive care, her hemodynamic status deteriorated rapidly, leading to multiple organ failure and death <24 hours after admission.

Necropsy showed hemorrhagic foci in both lungs. Histopathologic studies showed total destruction of the respiratory epithelium covering the bronchi and bronchioli, together with abundant gram-positive cocci, absence

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of viable polymorphonuclear cells, and extensive necrosis of the alveolar septa. These lesions were characteristics of necrotizing pneumonia.

S. aureus was isolated 3 times, by culture of blood (LIM-48), lung necropsy tissue (LIM-49), and tracheal aspirate (LIM-50). The 3 isolates had the same antimicrobial drug resistance profile (resistant to oxacillin, kanamycin, tobramycin, and gentamicin) and the same pattern by pulsed-field gel electrophoresis after DNA digestion by *Sma*I (data not shown).

Influenza A virus was detected by polymerase chain reaction and immunofluorescence on the tracheal aspirate, but culture on cell lines was negative. The influenza A virus has been shown to be capable of destroying the respiratory epithelium from the trachea to the bronchioli (*1*), allowing the PVL-positive CA-MRSA to adhere to the basement membrane (*4*). PVL may diffuse locally, attracting polymorphonuclear leukocytes by its chemotactic activity and lysing them by its leukotoxicity.

Because the clinical manifestations corresponded to those of necrotizing pneumonia, isolate LIM-49 was referred (lung necropsy culture) to the French National Centre for Staphylococci. The isolate was screened for staphylococcal toxin genes, *agr* alleles, and the *mecA* gene as described elsewhere (Table) (*3,5,6*). The strain had an *agr* type 1 allele and the *lukS-PV*/*lukF-PV* genes encoding PVL (Figure), but harbored no toxin genes (*sea* through *see*, *tsst*, *eta*, and *etb* genes encoding staphylococcal enterotoxins A though E, toxic shock syndrome toxin, and exfoliative toxins A and B, respectively). The *mecA* gene was detected, together with SCC*mec* type V as described by Ito et al. (*7*). The spa type was 355 and the sequence type was ST377 (Figure). The single nucleotide difference between ST152 and ST377, which is not distinguished by using the usual primers determined by Enright et al. (*8*), was established as ST377 by using in-house primers (forward 5′-ggA CgA Agg TCA TgA TgT ATT TTT-3′ (nt 327–350), reverse 5′-CTT CTA CgC gCT CTC TTT TTA Ag-3′ (nt 564–586), according to the GenBank ID11211186 of the *gmk* gene) to amplify appropriate fragment. To our knowledge, this is the first description of such a necrotizing pneumonia–associated CA-MRSA clone. Three other ST377 strains were referred to the National Reference Centre for staphylococci in 2003. Two were isolated from patients in Europe (Netherlands and Switzerland), and 1 from an Australia patient (strain provided by G. Nimmo). The patient from the Netherlands had been treated for an abscess from which PVL-MRSA was cultured, and the Swiss patient had furunculosis. The 3 isolates harbored the SCC*mec* type V cassette, an *agr*1

Table. Primers used to screen for staphylococcal toxin genes, *agr* alleles, and *mecA* gene

Gene	Primer	Oligonucleotide sequence (5′–3′)	Reference
mecA	*mecA*-1	AAAATCGATGGTAAAGGTTGGC	(*6*)
	mecA-2	AGTTCTGCAGTACCGGATTTGC	
agr1	*agr*1-1	ATGCACATGGTGCACATGC	(*5*)
	*agr*1-2	GTCACAAGTACTATAAGCTGCGAT	
agr2	*agr*2-1	ATGCACATGGTGCACATGC	(*5*)
	*agr*2-2	TATTACTAATTGAAAAGTGCCATAGC	
agr3	*agr*3-1	ATGCACATGGTGCACATGC	(*5*)
	*agr*3-2	GTAATGTAATAGCTTGTATAATAATACCCAG	
agr4	*agr*4-1	ATGCACATGGTGCACATGC	(*5*)
	*agr*4-2	CGATAATGCCGTAATACCCG	
sea	*sea*-1	GAAAAAAGTCTGAATTGCAGGGAACA	(*3*)
	sea-2	CAAATAAATCGTAATTAACCGAAGGTTC	
seb	*seb*-1	ATTCTAATTAAGGACACTAAGTTAGGGA	(*3*)
	seb-2	ATCCCGTTTCATAAGGCGAGT	
sec	*sec*-1	GTAAAGTTACAGGTGGCAAAACTTG	(*3*)
	sec-2	CATATCATACCAAAAAGTATTGCCGT	
sed	*sed*-1	GAATTAAGTAGTACCGCGCTAAATAATATG	(*3*)
	sed-2	GCTGTATTTTTCCTCCGAGAGT	
see	*see*-1	CAAAGAAATGCTTTAAGCAATCTTAGGC	(*3*)
	see-2	CACCTTACCGCCAAAGCTG	
tsst	*tsst*-1	TTCACTATTTGTAAAAGTGTCAGACCCACT	(*3*)
	tsst-2	TACTAATGAATTTTTTTATCGTAAGCCCTT	
eta	*eta*-1	ACTGTAGGAGCTAGTGCATTTGT	(*3*)
	eta-2	TGGATACTTTTGTCTATCTTTTTCATCAAC	
etb	*etb*-1	CAGATAAAGAGCTTTATACACACATTAC	(*3*)
	etb-2	AGTGAACTTATCTTTCTATTGAAAAACACTC	
LukS-PV/lukF-PV	*pvl*-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	(*3*)
	Npvl-2	GCATCAASTGTATTGGATAGCAAAAGC	

Figure. Pulsed-field gel electrophoresis (PFGE) pattern and phylogenetic tree of 4 strains ST377. *SmaI* macrorestriction patterns were digitized and analyzed by using GelCompar II (Applied Maths, Kortrijk, Belgium) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages clustering. The scale indicates the level of pattern similarity. PVL, Panton-Valentine leukocidin; ST, sequence type

allele, the same toxin gene profile, and macrorestriction pattern, *spa* type, as our isolate (Figure).

Conclusions

We report the identification of a new, emerging, highly virulent PVL-positive CA-MRSA clone that harbors the SCC*mec* type V cassette. This tends to confirm the continuing emergence of new CA-MRSA clones. The new clone seems to have spread rapidly in Europe, because it has been detected in patients in the Netherlands, Switzerland, and now in France. Since necrotizing pneumonia is rapidly fatal, as in the patient described here, cases of PVL-positive CA-MRSA infection must be recognized rapidly, especially in case of methicillin resistance. The therapeutic value of intravenous immunoglobulin containing anti-PVL antibodies has been reported by Gauduchon et al. (*9*) but must be confirmed.

Dr Garnier is a medical microbiologist at the Limoges Teaching Hospital, France. His research interests include molecular bacterial identification and bacterial resistance.

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Canine Leptospirosis, United States, 2002–2004

George E. Moore,* Lynn F. Guptill,* Nita W. Glickman,* Richard J. Caldanaro,* David Aucoin,† and Lawrence T. Glickman*

The proportion of positive *Leptospira* microscopic agglutination tests for 23,005 dogs significantly increased from 2002 to 2004 (p<0.002) regardless of the positive cutoff titer used and was highest (p<0.05) for serovars Autumnalis and Grippotyphosa. The strongest positive serologic correlation (r = 0.72) was between serovars Autumnalis and Pomona.

Leptospirosis is a zoonotic disease with reservoirs in companion animals, livestock, and wild animals. More than 200 *Leptospira* serovars have been identified (*1*). Dogs are considered maintenance hosts for serovar Canicola, incidental hosts for other serovars, and are a potential source of infection for pet owners (*2*). Bivalent leptospirosis bacterins containing serovars Canicola and Icterohaemorrhagiae have been available for use in dogs since the 1960s. Despite use of these bacterins, canine leptospirosis diagnosed at US veterinary teaching hospitals has increased since 1990 (*3*). Case reports have attributed canine infection primarily to serovars Grippotyphosa, Pomona, Bratislava, and Autumnalis (*4–6*). Although human leptospirosis ceased to be a notifiable disease in 1994, outbreaks are still reported and infecting serovars in humans are antigenically related to the emerging serovars in dogs (*7,8*).

The referent method for serologic diagnosis of leptospirosis is the microscopic agglutination test (MAT). The serovar in which agglutination is detected at the highest dilution of serum is indicative of the infective serogroup, but cross-reactions between serovars is common (*9*). Laboratory databases are potentially useful for surveillance of zoonotic pathogens. Therefore, we determined the percentage of positive MAT results for leptospirosis for each seropositive state by using sera from dogs with suspected clinical leptospirosis that were submitted to a nationwide veterinary diagnostic laboratory. We also evaluated the statistical correlation of seropositivity between different serovars.

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The Study

The results of all leptospirosis MATs for dogs from January 2002 through December 2004 were obtained electronically from Antech Diagnostic Veterinary Laboratory (Los Angeles, CA, USA). Antech provides laboratory services to >18,000 US veterinary hospitals. The 7 *Leptospira* serovars included in the MATs were Canicola, Grippotyphosa, Icterohaemorrhagiae, Hardjo, Pomona, Autumnalis, and Bratislava. MAT results for each serovar were reported as the highest dilution of serum (1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, or ≥1:12,800) at which ≥50% agglutination of organisms occurred when compared with a control suspension.

Calculation of seropositivity was performed separately by using cutoff titers of ≥400, ≥800, or ≥1,600. The percentage of seropositive test results for each serovar was calculated both as the number of positive test results divided by the total number of tests performed and by the total number of positive test results with 95% confidence limits. The percentage of seropositive MAT results was calculated by state and year. Proportions for categoric variables were compared by using the χ^2 test for independence. A rank from 1 to 9 was assigned based on the serum dilution results. If 2 serovars had equivalent titers on a MAT for a dog, both serovars received the same rank score appropriate for that dilution. Correlation of seropositivity between all 2-way comparisons of serovars was by Spearman rank correlation. All calculations were performed by using SAS version 9.1.3 statistical software (SAS, Cary, NC, USA), and a p value ≥0.05 was considered significant. Tests that used paired sera from the same dog or tests repeated on the same dog at a different time could not be identified because patient identifiers were not included in the database. Therefore, a few individual dogs could have contributed >1 test to the dataset, but this possibility was considered uncommon.

During the study, 23,005 serum samples were submitted for a leptospirosis MAT, and ≈23,000 tests were performed for each of 5 serovars, namely Canicola, Grippotyphosa, Icterohaemorrhagiae, Hardjo, and Pomona (Table). Laboratory testing for serovars Autumnalis and Bratislava was initiated in 2003, and ≈11,600 tests were performed for each of these 2 serovars. The percentage of MATs that were positive significantly increased from 2002 to 2004 by using cutoff titers ≥400 (p<0.002), ≥800 (p<0.0001), or ≥1,600 (p<0.0001). At these 3 cutoff titers, the percentage of positive MAT results was greatest for serovars Autumnalis (9.1%, 6.5%, and 4.7%, respectively) and Grippotyphosa (6.4%, 4.9%, and 4.0%, respectively).

The proportion of positive MAT results attributable to serovars Canicola or Icterohaemorrhagiae declined as the cutoff titer increased, while it generally increased for serovars Autumnalis, Bratislava, Grippotyphosa, and

Table. Seropositivity for *Leptospira* serovars in dogs by the microscopic agglutination test using canine sera, 2002–2004*

Serovar	Total tests (n)	Positive test results					
		≥400		≥800		≥1,600	
		n	% (95% CL)	n	% (95% CL)	n	% (95% CL)
Autumnalis	11,621	1,059	9.11 (8.60,9.65)	755	6.50 (6.06,6.96)	549	4.72 (4.35,5.13)
Grippotyphosa	22,929	1,458	6.36 (6.05,6.68)	1,132	4.94 (4.66,5.23)	908	3.96 (3.71,4.22)
Bratislava	11,663	499	4.28 (3.92,4.66)	428	3.67 (3.34,4.03)	357	3.06 (2.76,3.39)
Pomona	22,937	906	3.95 (3.70,4.21)	716	3.12 (2.90,3.35)	575	2.51 (2.31,2.72)
Canicola	22,377	669	2.99 (2.77,3.22)	317	1.42 (1.27, 1.58)	134	0.60 (0.50,0.71)
Icterohaemorrhagiae	22,935	356	1.55 (1.40,1.72)	179	0.78 (0.67, 0.90)	79	0.34 (0.27,0.43)
Hardjo	22,937	40	0.17 (0.12,0.24)	22	0.10 (0.06, 0.15)	10	0.04 (0.02,0.08)

*CL, confidence limit.

Pomona (Figure 1). No consistent or distinct geographic pattern for positive MAT results was observed in the study (Figure 2), but seropositivity was greater in the midwest, south-central, and northwest regions of the United States.

Moderately strong positive correlation in seropositivity (r, 0.59–0.72) was present between serovars Autumnalis, Pomona, Grippotyphosa, and Bratislava, with the strongest correlation between serovars Autumnalis and Pomona. In contrast, weak positive correlation ($r = 0.36$) was found between serovars Canicola and Icterohaemorrhagiae, and each of these serovars was weakly correlated (r, 0.20– 0.33) with serovars Autumnalis, Pomona, Grippotyphosa, and Bratislava. All rank correlation coefficients were significant at $p<0.0001$. Serovar Hardjo was excluded from correlation analysis because of the small number of positive test results.

Conclusions

Positive leptospirosis MAT results in dogs may indicate natural infection due to direct or indirect contact with wildlife maintenance hosts or recent vaccination (*2*). However, titers >800 from vaccination are considered unlikely as postvaccinal titers wane rapidly (*10*) and most leptospiral bacterins available for dogs are bivalent for Canicola and Icterohaemorrhagiae, 2 serovars with low seropositivity in this study. Although the health and vaccination status of dogs from which sera were submitted was unknown, veterinarians most likely submitted samples for leptospirosis testing when they suspected leptospirosis based on clinical signs including vomiting, fever, lethargy, and anorexia.

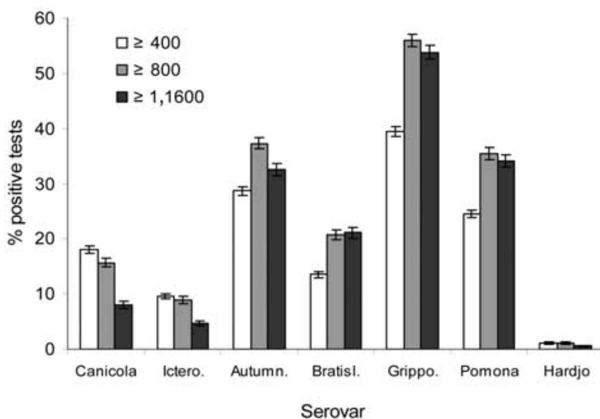

Figure 1. Percentage of positive microscopic agglutination tests by *Leptospira serovar*, using 3 different cutoff titers for 23,005 canine sera from 2002 to 2004. Serovars Canicola and Icterohaemorrhagiae have been used in canine bacterins for leptospirosis during the study period. Ictero., Icterohaemorrhagiae; Autumn., Autumnalis; Bratisl., Bratislava; Grippo., Grippotyphosa.

The most common serovar associated with a positive MAT result was Autumnalis, a serovar not currently included in licensed canine bacterins. Reactivity to this serovar in the MAT has been considered a possible paradoxical cross-reaction with serovar Pomona (*11*); a strong positive correlation in titers for these 2 serovars was found in this study. The Autumnalis serovar has been isolated from raccoons in the southern United States (*12*), and seropositivity in dogs may represent natural infection from this source. The MAT is not serovar-specific, but the 7 serovars evaluated in this study belong to different serogroups (*13*). Serovar Grippotyphosa, the second most common positive serovar in this study, has also been associated with human leptospirosis outbreaks in the 1990s (*8*).

The finding of a moderately high correlation in serologic reactivity between serovars Autumnalis, Pomona, Grippotyphosa, and Bratislava suggests that cross-protection to Autumnalis could be induced by current bacterins that lack this antigen. Canine vaccines are now available with serovars Grippotyphosa and Pomona as well as the traditional serovars Canicola and Icterohaemorrhagiae. This vaccine may confer some immunity to serovar Autumnalis, since some protein antigens are highly conserved among several pathogenic serovars (*14*).

Limitations of the present study included the inability to determine if multiple tests had been performed for individual dogs, lack of data on clinical signs, and unknown vaccination status of the dogs. The geographic distribution of serologic reactivity during the study, however, indicates broad dispersion of *Leptospira* pathogens that pose a risk to both domestic animals and humans.

Dogs in suburban or rural environments have been shown to be at increased risk of leptospirosis (*15*), presumably because of greater likelihood of contact with wildlife habitats. Dogs may be sentinels for human exposure to this zoonotic organism. Veterinary practitioners and public health officials need to be aware of the potential change in the ecologic environment and circulating endemic strains for this zoonotic organism.

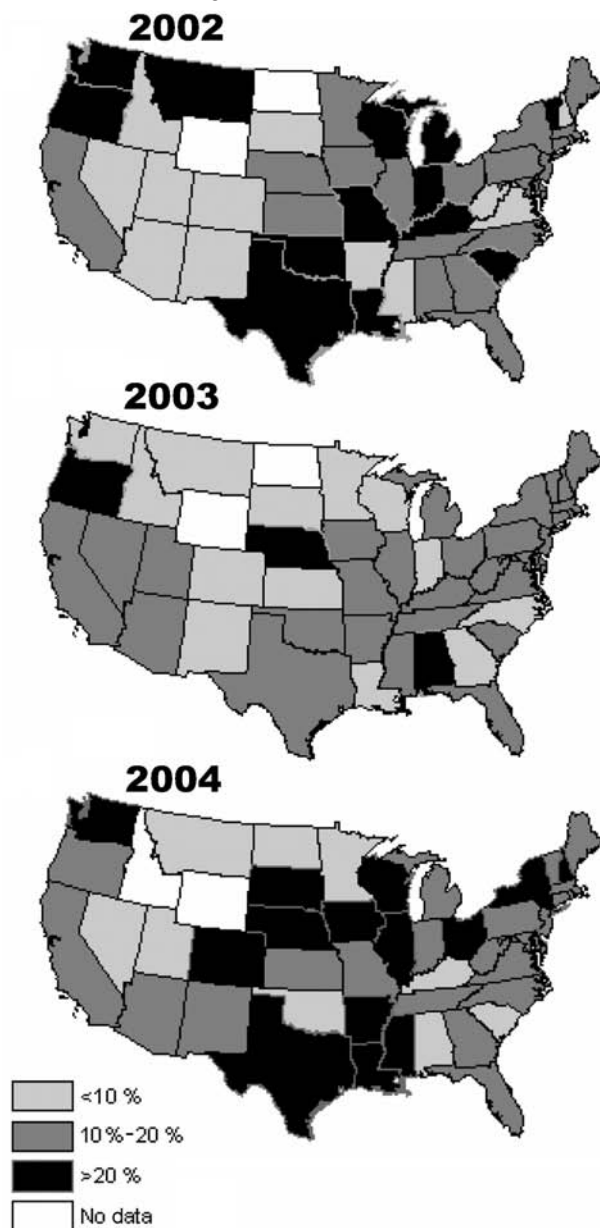

Figure 2. Canine leptospirosis microscopic agglutination test results shown as the percentage positive and standard error, by state and year from 2002 to 2004. A test was considered positive if the titer for any serovar was $\geq$400 for Autumnalis, Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona, or Hardjo serovars.

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Lagos Bat Virus, South Africa

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Three more isolates of Lagos bat virus were recently recovered from fruit bats in South Africa after an apparent absence of this virus for 13 years. The sporadic occurrence of cases is likely due to inadequate surveillance programs for lyssavirus infections among bat populations in Africa.

Since 2003, we have embarked on a passive surveillance study to collect and identify bats with neurologic disease signs that may indicate encephalitis due to lyssavirus infection. Consequently, 3 new cases of Lagos bat virus (LBV) infection in fruit bats were identified in South Africa, 1 each in 2003, 2004, and 2005. LBV is a member of the *Lyssavirus* genus in the *Rhabdoviridae* family. Rabies virus (RABV) was first isolated as a unique virus within this group. However, after the isolation of rabies-related viruses in Africa and Europe in the mid-1950s, the *Lyssavirus* genus was created, and rabies virus (genotype 1) was designated as the type-species member of the genus. At least 7 different major *Lyssavirus* species (genotypes) are recognized (*1*), but the genus will be expanded to include organisms isolated from Eurasia in recent years (*2*). At present, 4 *Lyssavirus* species (genotypes) are recognized in Africa. Of these, RABV (genotype 1) occurs worldwide, but LBV (genotype 2), Mokola virus (genotype 3), and Duvenhage virus (genotype 4) have not been encountered outside of Africa. Although RABV infection of bats is well known in the Americas, this virus has only been associated with infections of terrestrial mammals on the African continent. Mokola virus has also been isolated only from various terrestrial species, never bats (*3*). Both LBV and Duvenhage virus are thought to be bat viruses, although LBV infections of terrestrial animals have been reported (*4,5*). RABV is a zoonotic agent throughout Africa; Duvenhage virus and Mokola virus, but not LBV, have also been responsible for rare zoonotic events (*3,6*).

LBV was first isolated from a fruit bat in 1956 in Nigeria (*7*), but not until 1970 was it identified as a rabies-related virus (*8*). Since then (and before this report), 11 more isolations of LBV were made throughout Africa (Table 1), including 5 isolates from South Africa.

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The Case

In June 2003, an *Epomophorus wahlbergi* carcass was recovered in Durban, KwaZulu-Natal, after the bat was caught by a domestic cat. In August 2004, a resident of Umbilo, Durban, found a dead *E. wahlbergi* fruit bat on her lawn one morning after hearing squeaking noises around the house during the night. The fluorescent-antibody test (FAT), performed on brain material, was positive for lyssavirus antigens, and virus was isolated in both cases when suckling mice died 9–14 days after intracerebral injection with brain suspensions. Antigenic typing was carried out with a panel of anti-lyssavirus nucleocapsid monoclonal antibodies (prepared by the Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario, Canada). These analyses identified both new isolates as LBV (genotype 2) (Table 2). Additional characterization was accomplished by polymerase chain reaction (PCR) and sequencing of a 457-bp region of the nucleoprotein-encoding gene with a novel set of PCR and sequencing primers specific for LBV (LagNF (5′-GGGCAGATATGACGCGAGA-3′) and LagNR (5′-TTGACCGGGTTCAAACATC-3′). Briefly, total RNA was extracted from infected tissue by using TRIzol (Invitrogen, Croningen, the Netherlands) according to the manufacturer's instructions. Complementary DNA was produced by a reverse transcription reaction (RT) and used in subsequent PCR. PCR products were purified by using the Wizard SV PCR and Gel purification kit (Promega, Madison, WI, USA). The purified products were then sequenced by using the Big Dye Termination Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol, with subsequent analysis on an Applied Biosystems 377 DNA automated sequencer.

In June 2005, a caretaker/gardener at a communal outdoor sports complex in the Bluff, Durban, found a bat on the lawns of the complex. At the time, birds were picking at it, and on closer inspection, it was found to be an immobile adult animal with a pup attached to it. The caretaker collected both bats and placed them in a nearby tree. Later, the bats, still attached to each other, were again found on the ground, where eyewitnesses also saw a cat toying with it. The animals were then presented to a local bat rehabilitator. The adult animal died and was submitted for diagnostic testing, but results of FAT carried out on brain smears were repeatedly negative. The pup had at least 1 evident bite wound, presumably from the cat, but otherwise appeared healthy and was cared for by the rehabilitator. Although the pup was reported to be feeding and doing

Table 1. Lagos bat virus isolates recorded to date

Geographic origin	Year of isolation	Animal	Reference
Lagos, Nigeria	1956	Bat (*Eidolon helvum*)	(*7*)
Bozo, Central African Republic	1974	Bat (*Micropterus pusillus*)	(*9*)
Pinetown, South Africa (3 isolates)	1980	Bat (*Epomophorus wahlbergi*)	(*10,11*)
Stanger, South Africa	1982	Cat	(*11*)
Kindia, Senegal	1985	Bat (*Nycteris cambiensis*)	(*12*)
Dakar, Senegal	1985	Bat (*E. helvum*)	(*12*)
Dorowa, Zimbabwe	1986	Cat	(*4*)
Durban, South Africa	1990	Bat (*E. wahlbergi*)	(*6*)
Ethiopia	Before 1992	Dog	(*5*)
Egypt	1999	Bat (*Roussetus aegyptiacus*)	(*13*)
Durban, South Africa	2003	Bat (*E. wahlbergi*)	This report
Durban, South Africa	2004	Bat (*E. wahlbergi*)	This report
Durban, South Africa	2005	Bat (*E. wahlbergi*)	This report

well, it suddenly died ≈4 days after being found, on June 21, 2005. By this time, RT-PCR and nucleotide sequencing assays, carried out as described above, showed LBV in brain material from the adult. Antigenic typing was not performed because the level of lyssavirus antigen in the brain matter was undetectable. In the meantime, the carcass of the pup was recovered, and brain material was subjected to FAT and diagnostic RT-PCR. Although the RT-PCR results were inconclusive, the FAT results were negative.

DNA sequencing information from each case was compared with nucleoprotein sequence information for LBV and other lyssavirus species (genotypes) available in the public domain (GenBank). ClustalW was used to produce sequence alignments and generate a phylogenetic tree (Figure). A graphic representation of the trees was constructed with the TreeView program. In this phylogeny, the 3 new LBV isolates segregate together with previously identified LBV isolates from Ethiopia (AY333110) (*7*) and Nigeria (U22842) (*5*). The recent isolates from South Africa share a close sequence homology with the isolate from Ethiopia. This finding warrants further investigation.

Conclusions

Although LBV is rare and has not been reported in South Africa in 13 years, a small-scale passive surveillance effort in KwaZulu-Natal, South Africa, enabled us to identify 3 new isolations of LBV in a relatively short time. This finding reemphasizes our lack of understanding of the true prevalence of lyssaviruses in Africa because of poor surveillance for non-rabies viruses (and, in fact, RABV) throughout the continent. Human infections with LBV have not been documented to date; however, this virus has been reported in domestic animals (2 cats [*4*] and a dog [*5*]). We describe close contact between humans and other animals and LBV-infected bats. Cross-neutralization data obtained in rodent models show that rabies preexposure and postexposure prophylaxis is unlikely to be effective

Table 2. Immunofluorescence reaction of a panel of 16 monoclonal antibodies against the nucleoprotein of Lagos bat virus isolations, South Africa, 2003 and 2004*

Antibody	Canid biotype (GT1)	Mongoose biotype (GT1)	Lagos bat virus (GT2)	Mokola virus (GT3)	Duvenhage virus (GT4)	2003 isolate	2004 isolate
1C5	–	–	–	–	–	–	–
26AB7	+++	Variable	–	–	–	–	–
26BE2	+++	Variable	–	–	–	–	–
32GD12	Variable	Variable	–	–	–	–	–
38HF2	+++	+++	+++	+++	+++	+++	+++
M612	–	–	+++	–	–	+++	+++
M837	–	–	–	–	+++	–	–
M850	–	Variable	–	–	+++	–	–
M853	+++	–	–	–	+++	–	–
M1001	–	–	–	+++	–	–	–
M1335	–	Variable	–	Variable	–	–	–
M1386	–	+++	–	–	–	–	–
M1400	–	Variable	–	–	–	–	–
M1407	++	Variable	–	–	–	–	–
M1412	++	Variable	–	–	–	–	–
M1494	–	Variable	–	–	+++	–	–

*Typical immunofluorescence antibody pattern observed for all lyssavirus genotypes present on the African continent (genotype [GT] 1, 2, 3, and 4) are also included as a reference: –, no specific fluorescence; ++, strong fluorescence; +++, very strong fluorescence.

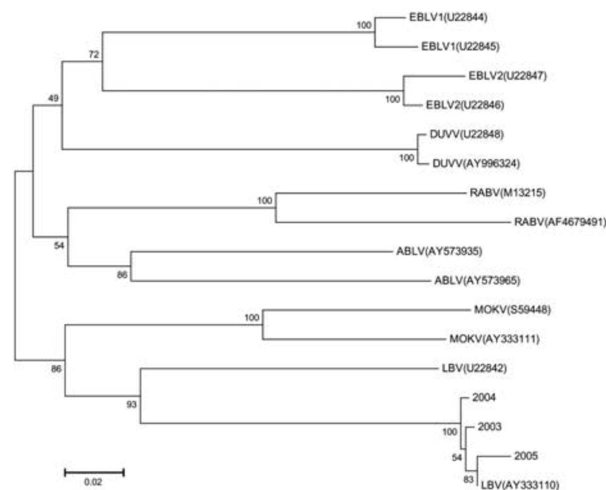

Figure. A neighbor-joining tree comparing 457 nucleotides of the nucleoprotein-encoding genes of the new Lagos bat isolations made in South Africa (bat 2003 [DQ201178], 2004 [DQ201179], and 2005 [DQ201180]) with representative sequences of the 7 genotypes of lyssaviruses obtained from GenBank. GenBank accession numbers are indicated on the figure. The bootstrap values were determined with 1,000 replicates.

against LBV (*14*). We have shown that LBV infection may be present in bat populations; consequently, we recommend appropriate precautions and use of proper personal protection equipment, such as gloves, when interacting with these animals. Even though the value of rabies vaccination is doubtful, it should be considered in light of the potential for cross-reactivity (*15*) and the lack of alternatives. Surveillance should be maintained as part of a strategy to better understand the epidemiology of LBV. Cumulatively, all available evidence indicates that LBV is likely persistently maintained in Megachiroptera populations in South Africa and other African countries where LBV has been reported in the past.

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Aquariums as Reservoirs for Multidrug-resistant *Salmonella* Paratyphi B

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Multidrug-resistant *Salmonella enterica* serovar Paratyphi B dT+ isolates from patients with gastroenteritis were identical with isolates from their home aquariums. Matched isolates had identical phage types, *Xba*I and IS*200* profiles, and *Salmonella* genomic island 1 (SGI1). Ornamental fish tanks are reservoirs for SGI1-containing *S.* Paratyphi B dT+.

Strains of *Salmonella enterica* serovar Paratyphi B that use d-tartrate as a carbon source (*S*. Paratyphi B dT+, formerly *S. enterica* serovar Java) primarily cause gastroenteritis (*1*). Since the late 1990s, multidrug-resistant *S*. Paratyphi B dT+ has been increasingly isolated from infected persons in different parts of the world. One type, which is resistant to streptomycin, spectinomycin, trimethoprim, and sulfonamides, carries a chromosomally located class 2 integron with the *dfrA1*-*sat1*-*aadA1* (Tn*7*) array of gene cassettes (*2*). This clone is predominantly associated with poultry and poultry products in Germany and the Netherlands (*2,3*). Human cases of gastroenteritis caused by *S*. Paratyphi B dT+ with the resistance phenotype ApCmSmSpSuTc (Ap, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamides; Tc, tetracycline) have also been found in Canada (*4*), the United Kingdom (*5*), France (*6*), and Australia (*7*), and their incidence is increasing. In most of the studied isolates, the resistance genes *blaP1*, *floR*, *aadA2*, *sul1*, and *tetG* are located in a complex class 1 integron recently designated In104 (*7*) (Figure 1). This integron is located within the *Salmonella* genomic island 1 (SGI1) that was first identified in *S. enterica* serovar Typhimurium DT104 strains with the same phenotype (*8*). However, the source of the SGI1-containing *S*. Paratyphi B dT+ has not been identified. Whether isolates obtained in different countries are clonally related is also not known.

Although a few epidemiologic studies suggest that antimicrobial drug–susceptible *S*. Paratyphi B dT+ may be linked to aquacultural practices (*9,10*), no molecular data confirm this. However, the first reported SGI1-containing *S*. Paratyphi B dT+ isolate with drug-resistance phenotype ApCmSmSpSuTc was isolated in 1997 from a tropical fish in Singapore (*11*), raising the possibility that tropical fish and aquariums are a reservoir. The aim of this study was to determine if domestic aquariums are reservoirs for SGI1-containing, multidrug-resistant *S*. Paratyphi B dT+ that infect humans.

The Study

S. Paratyphi B dT+ with the resistance phenotype ApCmSmSpSuTc had been isolated sporadically in various states of Australia since 1997, and initial surveys showed a potential association with ownership of home aquariums (D. Lightfoot, unpub. data). In 2000, multidrug-resistant *S*. Paratyphi B dT+ with an identical phage type (reaction does not conform [RDNC]), designated here as Aus2, and the same drug-resistance profile (ApCmSm SpSuTc) was isolated from humans with gastroenteritis and from fish tanks in the homes of 2 infected patients (Table). In 2003 and 2004, 13 cases of ApCmSmSpSuTc *S*. Paratyphi B dT+ were investigated by state and commonwealth health departments, and all were associated with home aquariums containing tropical fish (J. Musto et al., unpub. data). Of these, 11 cases were phage type RDNC Aus3, 1 was phage type 1 var 15, and 1 was phage type 3b var. Water and gravel were collected from the domestic

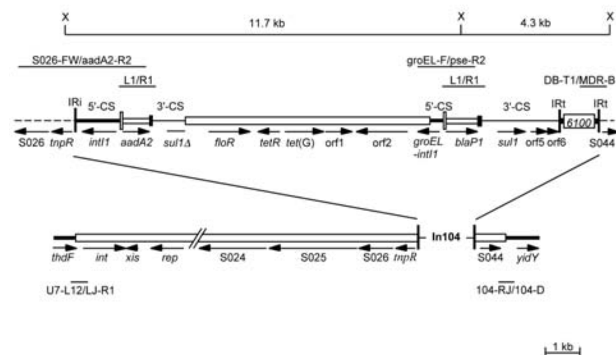

Figure 1. Structure of *Salmonella* genomic island 1 (SGI1). The SGI1 region of serovar Typhimurium DT104 (GenBank accession no. AF261825) is drawn to scale with In104 above. Different discrete segments are represented by open boxes and lines of different thicknesses. Vertical bars indicate the IR bounding In104. The chromosomal genes *thdF* and *yidY* flank SGI1. Fragments amplified by polymerase chain reaction are shown as thin lines with the primer pairs indicated. Sequences of primers have been previously described (*7*). Arrows indicate the position and orientation of genes and open reading frames. The positions of *Xba*I sites (×) are indicated with the fragment sizes shown.

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Table. Genetic characteristics of *Salmonella enterica* serovar Paratyphi B dT+ isolates used in this study

Isolate no.*	Source†	Phage type‡	State§	Date of isolation	Age, y/Sex	SGI1¶
Set 1						
SRC229	H	Aus2	ACT	2000	<1/F	+
SRC230	H	Aus2	ACT	2000	1/M	+
SRC231	FT	Aus2	ACT	2000	–	+
Set 2						
SRC232#	H	Aus2	Vic	2000	11/F	+
SRC233#	H	Aus2	Vic	2000	11/F	+
SRC233A	FT	Aus2	Vic	2000	–	ND
Set 3						
SRC145	H	Aus3	Vic	2003	74/F	+
SRC142	FT	Aus3	Vic	2003	–	+
SRC143	FT	Aus3	Vic	2003	–	+
Set 4						
SRC149	H	Aus3	Vic	2003	12/M	+
SRC147	FT	Aus3	Vic	2003	–	+
SRC148	FT	Aus3	Vic	2003	–	+
Control						
SRC50	H	RDNC	Vic	2001	14/M	+**

*All isolates were resistant to ampicillin, chloramphenicol, streptomycin, spectinomycin, sulfonamides, and tetracycline.
†H, human isolates; FT, fish tank isolates.
‡Determined by using standard procedures and designations (http://www.geocities.com/avinash_abhyankar/typing.htm). RDNC, reaction does not conform. RDNC Aus2 and RDNC Aus3 are 1 var and 3b var phage-typing variants, respectively, and are identifiable and reproducible phage-typing patterns awaiting formal assignment by the World Health Organization–designated International Reference Laboratory, Colindale, UK.
§ACT, Australian Capital Territory; Vic, Victoria.
¶SGI1, *Salmonella* genomic island 1; ND, strain not available for molecular analysis.
#Isolates are from the same person.
**Data from Levings et al. (*7*).

aquariums of 5 patients with RDNC Aus3-type infections, and identical isolates were recovered from each fish tank. Four matched sets of isolates, 2 from 2000 and 2 from 2003, were further examined (Table). One isolate (SRC50) characterized previously (*7*) was used as a control (Table).

To determine if the resistance phenotype of these strains was due to SGI1 (*4,5,7,8,11*), polymerase chain reaction (PCR) with primer pairs shown in Figure 1 was used as previously described (*7*). The left and right junctions of SGI1 with the chromosome and of In104 with SGI1 were present in all cases. Regions containing the gene cassettes were amplified by using standard primers (L1 and R1) in the 5′- and 3′-conserved segments of class 1 integrons. Fragments of 1.0 and 1.2 kb were amplified from all isolates, and digestion of these amplicons with *Rsa*I generated a profile (data not shown) that was indistinguishable from the pattern for the 2 amplicons containing the *aadA2* and *blaP1* cassettes found in In104 and *S*. Paratyphi B dT+ isolates SRC49 and SRC50 from 2001 (*7*). The *aadA2* gene cassette was linked to SO26 in the SGI1 backbone, which indicates that it is on the left, as in In104, and the expected 1.8-kb PCR fragment was generated by using primers in *groEL* and *blaP1* (Figure 1), which places the *blaP1* cassette on the right. Southern hybridization of *Xba*I–digested whole-cell DNA with a probe for the *floR* gene as described previously (*7*) identified a band of ≈12 kb, which is consistent with an SGI1 structure identical to that reported previously (*7,8,11*) and the *groEL-blaP1* amplicon linked this 12-kb *Xba*I fragment with the adjacent 4.3-kb *Xba*I fragment (Figure 1).

To obtain further evidence for the identity of the matched human and fish tank isolates, macrorestriction analyses of *Xba*I-digested whole-cell DNA by pulsed-field gel electrophoresis (PFGE) were performed as previously described (*12*). Several studies (*3–6,13*) suggest that *S*. Paratyphi B dT+ isolates possess considerable genetic heterogeneity. However, the SGI1-containing isolates appear to be homogeneous. The band patterns for all SGI1-containing *S*. Paratyphi B dT+ were identical from humans and fish tanks with phage type RDNC Aus3 (Figure 2A) and Aus2 (data not shown). IS*200* profiles were also analyzed by hybridization of an IS*200* probe with *Pst* I-digested whole-cell DNA as described elsewhere (*6*). Again, all strains showed identical profiles (Figure 2B and data not shown) that differed by 1 band from profile IP1 recently described (*6*). Thus, matched isolates from humans and their fish tanks were indistinguishable from each other.

An unusual observation in this study was that isolates with different phage types showed identical PFGE and IS*200* profiles, indicating that they represented a clonal cluster. The control strain SRC50 (RDNC) also displayed the same patterns, demonstrating that it also is a member of the same clone. Thus, variation in phage type (Table) appears to have occurred within a single clone. Variation in phage type has also been reported in other studies of multidrug-resistant *S*. Paratyphi B dT+ strains (*4–6*), although

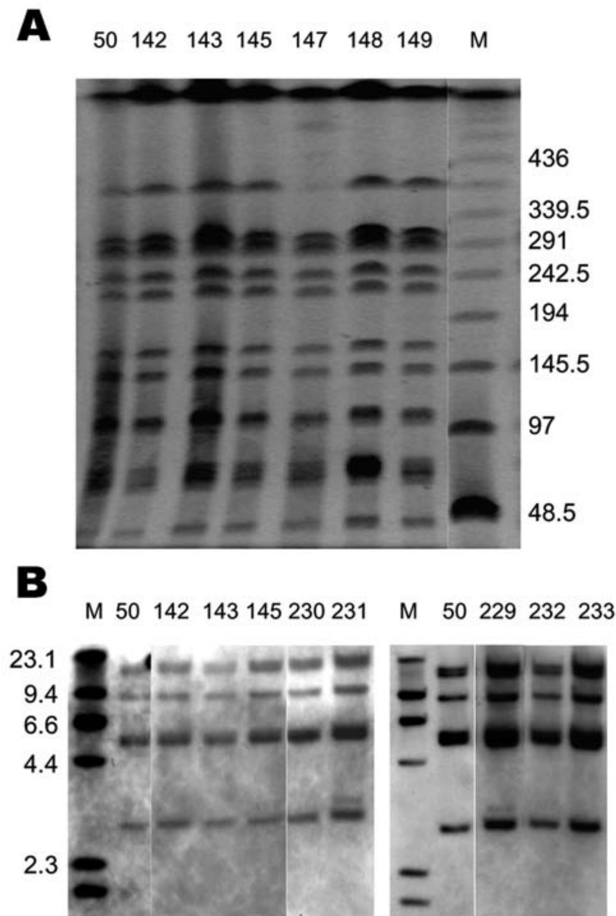

Figure 2. Pulsed-field gel electrophoresis (PFGE) and IS*200* profiles of *Salmonella enterica* serovar Paratyphi B dT+ isolates positive for *Salmonella* genomic island 1. A) PFGE profiles. *Xba*I–digested whole-cell DNA was separated by PFGE as previously described (*12*). Molecular mass markers (lane M) are low-range PFGE markers (New England BioLabs, Beverly, MA, USA) composed of concatamers of bacteriophage lambda DNA. The band absent in lane 147 was present in other runs. B) IS*200* profiles. *Pst*I digests of whole-cell DNA were separated and hybridized with an IS*200* digoxigenin (DIG)–labeled probe. Molecular mass markers (lane M) are DIG-labeled bacteriophage lambda DNA digested with *Hind*III (Roche Diagnostics, Castle Hill, New South Wales, Australia). Primers and polymerase chain reaction conditions used to generate the IS*200* probe have been previously described (*6*).

a number of related but slightly different *Xba*I PFGE patterns were observed in those studies. This finding suggests that all multidrug-resistant *S.* Paratyphi B dT+ found globally have a single origin, but that variations, possibly because of acquisition of other temperate phages or plasmids, have arisen over time. However, direct comparisons of strains from different countries will be needed to confirm this hypothesis.

Conclusions

This is the first definitive report showing that ornamental fish tanks are a reservoir for multidrug-resistant *S.* Paratyphi B dT+ (ApCmSmSpSuTc phenotype) containing SGI1 that causes severe disease in humans, particularly young children. In addition to containing SGI1, the matched isolates from humans and their fish tanks had the same phage type and the same *Xba*I macrorestriction digest pattern and IS*200* profile. These findings identify home aquariums containing tropical fish as the most important, although not necessarily the only, source of multidrug-resistant *S.* Paratyphi B dT+. The fact that 12%–14% of Australian households have ornamental fish (*14*) and as many as 12 million American and 1 million Canadian families own domestic aquariums (*9*), together with the young age of most affected patients, indicate that multidrug-resistant *S.* Paratyphi B dT+ in home aquariums is a risk factor for *Salmonella* infection and thus becomes a public health issue.

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Protease-resistant Prion Protein in Lymphoreticular Tumors of Variant Creutzfeldt-Jakob Disease Mice

Larisa Cervenakova,* Oksana Yakovleva,* and Carroll McKenzie*

We report protease-resistant prion protein (PrP^{res}) in spontaneous lymphoreticular tumors of mice infected with the agent of variant Creutzfeldt-Jakob disease (vCJD). PrP^{res} may accumulate in lymphoreticular system tumors of asymptomatic persons with vCJD. The statistical power of estimates of vCJD prevalence might be increased by expanding screening to include samples of lymphoreticular neoplasms.

Variant Creutzfeldt-Jakob disease (vCJD) is thought to be caused by exposure to bovine products contaminated with the bovine spongiform encephalopathy agent. The prevalence of preclinical and subclinical vCJD in the United Kingdom and other European countries is still unknown. To date, all tested vCJD patients have shown an accumulation of misfolded protease-resistant protein (PrP^{res}), a highly reliable indicator of infection, in lymphoreticular tissues such as spleen, tonsil, lymph nodes, and appendix (*1*). Although the time PrP^{res} starts to appear in lymphoreticular tissues of infected persons is unclear, it has been found in appendixes of 2 persons 8 months and 2 years before vCJD developed (*2*), in a lymph node and the spleen of a patient who died from a nonneurologic disorder 5 years after receiving a blood transfusion from a donor in whom vCJD subsequently developed (*3*), and in the appendixes of 3 persons from a large retrospective population study (*4*).

Lymphoreticular accumulation of infectivity and PrP^{res} occur early after scrapie infection in sheep and in various experimental animal models of transmissible spongiform encephalopathies, including mice infected with the vCJD agent (*5*). The presence of infectivity and PrP^{res} in inflamed liver, pancreas, and kidney tissues has been recently observed in transgenic and spontaneous mouse models of chronic inflammation on infection with the Rocky Mountain Laboratory strain of scrapie (*6*), and PrP^{res} has been shown in mammary glands of scrapie-infected sheep with mastitis (*7*). We report the first observation of PrP^{res} in spontaneous lymphoreticular tumors of mice with vCJD.

The Study

Experimental studies in mice were approved by the institutional animal care and use committee of the American Red Cross Holland Laboratory. Ten inbred, 7-week-old SJL/OlaHsd (Harlan, Bicester, UK) female mice closely related to the SJL/J strain, which develops spontaneous B-cell lymphomas at $\geq$8 months of age (*8,9*), were intracerebrally injected under isoflurane anesthesia with 1% vCJD human brain homogenate (World Health Organization reference material) (*10*) diluted in physiologic saline, while 4 control animals received physiologic saline only. Approximately 6 months after infection, visible tumors developed in the neck areas of 5 mice, 4 with vCJD and 1 control. Two of the vCJD animals were euthanized on day 199 because of rapid tumor growth (Table). The remaining mice in the vCJD group, including 2 other animals with tumors, were later euthanized or died (range 222–386 days) without noticeable signs of neurologic disease.

In the control group, the animal with tumors was euthanized on day 321, and the 3 other animals without tumors were euthanized on day 405. The autopsy of all mice, infected or not, revealed hepatomegaly and splenomegaly, with various degrees of white, nodular infiltrations of the spleen. Mice with visible tumors also had massive neoplastic nodular involvement of intestinal, mesenteric, cervical, and axillary lymph nodes and thymus. Brains and spleens were removed from all mice, and neoplastic tissues involving lymph nodes were removed from 4 infected and 1 uninfected mouse, and the thymus was removed from 2 infected mice (Table). Organs were sectioned, immediately frozen on dry ice, and stored at –80°C. PrP^{res} was extracted from brains by using high-speed centrifugation, from spleens by using methanol precipitation according to previously described methods (*5*), and from tumors with the procedure applied to the brain. Western blotting (WB) was performed by using PrP^{res}-specific monoclonal antibody 6H4 (Prionics, Schlieren, Switzerland) or 6D11 as previously described (*5*).

In the vCJD group, PrP^{res} was identified in the brains and spleens of all 10 mice. In 4 mice with tumors, PrP^{res} was found in neoplastic tissues of lymph nodes and also in the neoplastic thymus of 2 of the mice (Table). The Figure shows WB analysis of PrP^{res} extracted from the brain of a vCJD patient and representative tissues of a vCJD mouse with tumors. The glycosylation pattern of PrP^{res} in mouse tissues was typical of vCJD; diglycosylated isoforms predominated over monoglycosylated and unglycosylated

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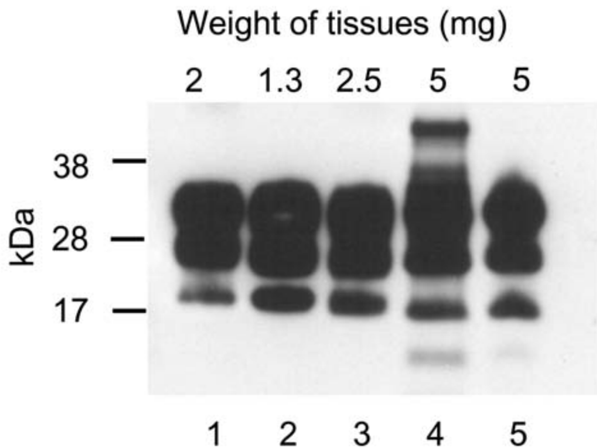

Figure. Immunoblot for protease-resistant prion protein (PrP^{res}) from tissues of SJL/OlaHsd mouse infected with human variant Creutzfeld-Jakob disease (vCJD). Lanes 1–5 show representative pattern of extracted PrP^{res} after digestion with proteinase K (100 µg/mL). Lane 1, brain tissue of vCJD patient (World Health Organization reference sample). Lanes 2–5, samples from vCJD mouse in which spontaneous lymphoreticular system tumors developed: lane 2, brain; lane 3, spleen with nodular tumors; lane 4, tissue from neoplastic lymph nodes; lane 5, neoplastic thymus. The amount of original tissue used for PrP^{res} extraction is shown on the top. Samples were denatured by boiling for 10 min in Laemmli buffer containing 2% β-mercaptoethanol, resolved on NuPAGE 12% Bis-Tris gel (Invitrogen Life Technologies, Carlsbad, CA, USA), transferred to nitrocellulose membrane, and probed with anti-PrP monoclonal antibody 6D11 (dilution 1:5,000). Major glycoforms of PrP^{res} are present as 3 bands corresponding to diglycosylated, monoglycosylated, and unglycosylated molecules.

isoforms, with the unglycosylated isoform corresponding to a 19-kDa fragment. On the basis of WB band intensity, we observed that the concentration of PrP^{res} in neoplastic lymphoreticular tissues (lanes 3–5) was similar to that seen in the human (lane 1) and mouse (lane 2) brains. Among the control mice, PrP^{res} was not detected in the brain and spleen of any animal or in neoplastic tissues of the single affected animal (Table).

Conclusions

Using immunohistochemical (IHC) tests, Hilton and colleagues (*1*) showed widespread PrP^{res} accumulation in the lymphoreticular system of 54 vCJD patients but not in 56 patients with familial or sporadic CJD. In contrast, when sodium phosphotungstate concentration for PrP^{res} was used to increase the sensitivity of the WB, PrP^{res} was detected in spleens of ≈30% of patients with sporadic CJD (*11*). A similar high-sensitivity detection method was used to screen 2,000 tonsils from the general population in a recently reported prospective study, with a negative result (*12*). The same method did not show PrP^{res} in the tonsils and 1 lymph node of an 83-year-old person who died from nonneurologic disease but who, 5 years before death, received a blood transfusion from a person in whom vCJD later developed (*3*, R. Will, pers. comm.). However, another cervical lymph node of this person tested positive for PrP^{res} by IHC test, although the appendix tested negative. This observation suggests that large retrospective and prospective studies based on screening of appendixes and tonsils with WB may not detect persons who have PrP^{res} in their lymph nodes. Estimates of prevalence of persons infected with the vCJD agent in the UK population may have been biased as a consequence of specimen selection from mostly younger participants. A retrospective study of

Table. Demonstration of protease-resistant prion protein (PrP^{res}) in the brain and lymphoreticular tissues of SJL/Ola mice infected with the agent of variant Creutzfeldt-Jakob disease (vCJD)

		Western blot (PrP^{res})*		
			Lymphoreticular tissue	
Experimental group	Postinfection interval (d)	Brain	Spleen	Lymph node
Mice infected with vCJD agent				
1*	199	+	+	+†
2*	199	+	+	+†
3	222	+	+	Not done
4*	318	+	+	+
5	318	+	+	Not done
6	318	+	+	Not done
7	342	+	+	Not done
8*	343	+	+	+
9	382	+	+	Not done
10	386	+	+	Not done
Control mice injected with 0.9% NaCl				
1	405	–	–	Not done
2	405	–	–	Not done
3*	321	–	–	–
4	405	–	–	Not done

*Widespread tumors of lymphoreticular tissue developed.
†Mouse also had PrP^{res} in tumor involving thymus.

>8,000 specimens of appendixes and tonsils included ≈70% from persons 20–29 years of age (*2*), and in a prospective study, approximately half the tonsillectomy samples came from children <9 years of age (*12*).

Our observation of the widespread presence of PrP^{res} in neoplastic lymph nodes of mice infected with the vCJD agent, and its absence in an uninfected mouse, provides experimental evidence that such tissues could be a valuable source for screening for vCJD in humans. The finding of unusually high amounts of PrP^{res} in neoplastic lymphoreticular tissues of vCJD mice, in a range comparable to that of the human and mouse brain, suggests that rapidly growing lymphoreticular tumors accumulate PrP^{res} at a high rate. Therefore, PrP^{res} might be detected in neoplastic lymphoreticular tissues of persons with vCJD. This finding is of particular importance because a recent UK study of samples collected before 1986, the years preceding the vCJD epidemic, found no PrP^{res} in lymph nodes from 58 patients with reactive conditions and 21 patients with lymphomas and carcinomas (*1*), which indicates that PrP^{res} does not spontaneously accumulate in tumors of uninfected persons. Whether PrP^{res} starts to accumulate in lymph nodes before it appears in spleens, appendixes, or tonsils of persons infected with the vCJD agent is not known. In vCJD mice, we observed PrP^{res} in the brain and neoplastic spleens and lymph nodes during at least half of the incubation period (199 days) when compared to mice with the longest survival time (>380 days). On the basis of our findings, we propose that screening of lymph node tissues from persons with reactive and neoplastic conditions and patients with various cancers with metastases in lymphoreticular organs could provide additional information, especially regarding older persons, on the prevalence of vCJD in the United Kingdom and other European countries.

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West Nile Virus–associated Flaccid Paralysis Outcome

James J. Sejvar,* Amy V. Bode,† Anthony A. Marfin,† Grant L. Campbell,† John Pape,‡ Brad J. Biggerstaff,† and Lyle R. Petersen†

We report 1-year follow-up data from a longitudinal prospective cohort study of patients with West Nile virus–associated paralysis. As in the 4-month follow-up, a variety of recovery patterns were observed, but persistent weakness was frequent. Respiratory involvement was associated with considerable illness and death.

During the summer and fall of 2003, we conducted a prospective, population-based study among residents of Larimer, Weld, and Boulder Counties in northern Colorado in whom West Nile virus (WNV)–associated paralysis developed (*1*). We identified 32 patients with paralysis and acute WNV infection. Clinical or neurodiagnostic findings suggested a poliomyelitis-like syndrome in 27 (84%) and a Guillain-Barré–like syndrome (GBLS) in 4 (13%); 1 patient had brachial plexus involvement alone. The cohort was reevaluated 4 months later, at which time 3 patients with respiratory failure had died, 2 remained intubated, 25 showed varying degrees of improvement, and 2 were lost to follow-up. Here, we describe the results of a 1-year follow-up evaluation of the 27 remaining cohort members and describe the patterns of recovery, persistence of symptoms and signs, and long-term outcome.

The Study

By 1 year, 3 of the 27 remaining cohort members had died (all with respiratory involvement), and an additional 2 persons (1 with poliomyelitis-like syndrome, 1 with GBLS) were lost to follow-up, leaving 22 patients in the 1-year cohort. Various degrees of strength improvement by manual muscle testing (MMT) using the Medical Research Council (MRC) 1–5 scale (*2*) were seen in the 18 of 27 patients with poliomyelitis-like syndrome at 1 year (online Appendix Figure, available at http://www.cdc.gov/ncidod/EID/vol12no03/05-0643-G.htm). Greater gains in MMT scores were seen between strength nadir and 4 months than between 4 months and 1 year by both subjective patient description and by serial MMT. Using a proportional odds model (SUDAAN, version 9.0.1, Research Triangle Institute, Research Triangle, NC, USA), we found that MMT scores improved over the 3 evaluations ($p<0.001$, adjusting for a significant MMT site effect), with the odds ratio for 4 months (relative to the nadir) of 0.40 (95% confidence interval [CI] 0.25–0.63) and for 1 year of 0.23 (95% CI 0.14–0.36), supporting subjective and objective assessment. Facial weakness, which had been present in 10 patients, had completely resolved in all patients by the 1-year follow-up.

One person (patient 27, Appendix Figure) who initially had quadriplegia and respiratory involvement had returned to pre-illness strength and had no detectable weakness on MMT at 1-year follow-up. Four persons (3 with tetra- or quadriplegia [patients 14, 15, and 31] and 1 with monoplegia [patient 37]) had achieved near-baseline strength (trace weakness on MMT) in affected limbs and reported little or no functional difficulty. Between the 4-month and 1-year follow-up, 4 persons (patients 3, 9, 16, 21) experienced measurable continued improvement between 4 months and 1 year (improvement in MMT score in at least 1 affected limb by $\geq$1 scale points), and 9 persons (patients 1, 7, 17, 20, 29, 34, 36, 41, 42) experienced little or no subsequent improvement. Four patients (7, 17, 29, 37) had a 1-point strength decrease in certain muscle groups and specific ranges of motion on MMT between 4 months and 1 year; however, overall strength for major joint ranges of motion or the entire limb did not decrease. All patients employed before illness onset (n = 11) had returned to work, 9 full-time and 2 part-time. Two persons were still using ambulatory aids (canes); 2 others continued to use leg braces.

Persistent symptoms and neurologic signs were observed in 10 persons (Table). Dependent edema and skin changes in affected limbs not present at 4 months were observed in 6 patients. In 1 patient, a deep venous thrombosis of an affected monoplegic leg had developed, presumably because of lack of leg movement. Fatigue was the most commonly reported subjective symptom (11, 56%), followed by myalgias/body pains (6, 27%), anxiety and depression (5, 23%), and mental/cognitive complaints and muscle cramps (4, 18% each).

One person with GBLS was lost to follow-up. Of the remaining 3 patients, 2 (patients 10, 32) had returned to baseline in terms of strength and overall functioning (Appendix Figure). The patient with brachial plexopathy and associated isolated shoulder abduction weakness had no additional strength gains in affected muscle groups at 1 year but continued to be unhampered functionally by this deficit.

By the 1-year follow-up, an additional 3 patients with respiratory failure had died (at 4.5 months, 5 months, and 7 months, respectively, after symptom onset). Of the 6

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Table. Signs and symptoms in 32 patients with West Nile virus (WNV)–associated paralysis

Sign/symptom	Acute infection, N = 32, no. (%)	4-mo followup, N = 27, no. (%)	1-y followup, N = 22, no. (%)
Fever (temperature ≥38°C)	29 (91)	0	0
Nausea (with or without vomiting)	26 (81)	0	0
Headache	28 (88)	5 (19)	3 (11)
Altered mental status	16 (50)	0	1 (5)
Meningismus	10 (31)	0	0
Rash	4 (13)	0	0
WNV-associated neurologic features*			
Tremor	21 (66)	8 (25)	9* (41)
Myoclonus	15 (47)	2 (6)	3* (14)
Parkinsonism	8 (25)	2 (6)	5* (23)
Cerebellar ataxia	3 (9)	2 (6)	1 (5)
Limb atrophy	0	17 (53)	10 (45)

*An apparent increase in the number of persons with tremor, myoclonus, and parkinsonism between 4 mo and 1 y is reflective of detection of these movement disorders in persons who were initially flaccid/immobile, nonambulatory, or too functionally impaired to assess.

deaths, cause of death was withdrawal of ventilatory support in 5 and cardiac arrest in 1. No persons intubated for ≥4 months survived. All 6 surviving patients had been discharged from care facilities and were living at home. One patient with GBLS and 1 with poliomyelitis-like syndrome were lost to follow-up by 1 year. Four of the 6 survivors required repeated episodes of extubation and reintubation before being permanently extubated. Of the 4 patients evaluated at 1 year, 2 still required assistance with all daily activities and required supplemental oxygen. The other 2 patients experienced substantial improvements in strength and functional ability, living independently and working at 1 year; however, both continued to experience persistent parkinsonism, tremor, and fatigue. All patients with respiratory involvement continued to experience orthopnea, dyspnea on exertion, and weakness of cough.

Of the 5 patients who experienced shortness of breath and had diagnostic evidence of respiratory muscle dysfunction but did not require intubation, 2 required supplemental oxygen at some point during their recovery. At 1 year, 3 patients continued to experience substantial orthopnea and dyspnea on exertion, and 2 had no respiratory symptoms.

Conclusions

The 1-year follow-up findings among this cohort of persons with WNV-associated paralysis demonstrate a spectrum of functional and strength outcomes. Among survivors, all demonstrated at least some improvement in strength of affected muscles, but improvement varied substantially. Consistent with findings at 4 months, those persons with less profound initial weakness demonstrated the greatest strength gains at 1 year. Strength gains among patients were more substantial between the nadir and 4 months than in the following months, in which a plateau was reached and less improvement was noted. The MRC scale of MMT is not linear, and this slowing of improvement may be attributable to the inherent nature of the MMT scoring system. However, this pattern of improvement is consistent with observations of recovery from poliovirus infection, in which most strength gains occur within 6 months (*3*). Persons with GBLS experienced complete or near complete recovery, a commonly reported outcome of this syndrome (*4*). Facial weakness was associated with favorable prognosis; however, resolution of weakness took ≥5 months in some cases.

Respiratory involvement was associated with considerable death and illness; the death rate was 50%. With the exception of 1 patient, death was due to a voluntary withdrawal of ventilatory support. In 2 of these patients, cognition and awareness were intact at the time of support withdrawal. Successful extubation among surviving patients with respiratory involvement occurred only after prolonged weaning periods (mean duration of intubation 66 days) (*1*) and often, multiple episodes of extubation and reintubation. At the 1-year follow-up, 2 of the surviving patients with respiratory involvement had severe disability and required assistance with all activities. However, 2 others with initial respiratory involvement did well; 1 experienced complete strength recovery and the other experienced recovery to the point of functional independence. Predictors of favorable outcome are unknown, but the 2 persons with favorable outcome were relatively young (45 and 43 years of age, respectively) and had no preexisting medical conditions. In contrast, all but 1 person who died or had a poor outcome were >56 years of age, and 4 had noteworthy prior medical conditions. Only 1 of the 5 patients with respiratory paralysis and quadriplegia from poliomyelitis-like syndrome survived, but that patient made a full and complete recovery; thus, respiratory paralysis and quadriplegia do not appear to universally indicate poor outcome. All persons recovering from respiratory failure continued to describe mild persistent orthopnea and exertional dyspnea. Respiratory paralysis appears

to be a long-term manifestation of WNV poliomyelitis-like syndrome associated with substantial morbidity.

Although small decreases in strength of specific movements at specific joints were noted in a few persons between 4 months and 1 year (Appendix Figure), these decreases were minor and observed in isolated ranges of motion, and most likely reflected a combination of MMT measurement variability, patient effort, and the method used to calculate strength scores. Only 1 person displayed worsening in strength of a major joint or limb after initial improvement; a decrement in strength from the 4-month time point was noted in the left upper arm of patient 15 because of limitation of testing of this limb from shoulder arthritis pain. A relapse or subsequent worsening in strength after an initial period of improvement was not observed in these patients, although such phenomena have been observed in patients with poliovirus infection (*5*). The possibility of a "postpoliomyelitis syndrome" (*6*) (e.g., development of weakness in a previously affected limb years after recovery) is unknown and will require longer term assessment.

Persistent associated neurologic signs and symptoms were seen or reported in nearly all patients (Table). Atrophy of affected limbs was common. Dependent edema, presumably due to compartmental muscle weakness and inability to augment blood circulation by muscular contraction, had been present to a mild degree in 2 persons at 4-month follow-up but was seen in 6 persons at 1 year. The development of a deep venous thrombosis in 1 person with leg paresis reinforces the need for vigilance for this complication in persons with severe weakness and lack of mobility.

In summary, the longer-term outcome of WNV poliomyelitis-like syndrome appears to be more heterogeneous than preliminary data may have suggested, with some persons experiencing little neurologic and functional improvement and others experiencing substantial gains. The degree of initial weakness appears to be an indicator of subsequent long-term outcome. WNV poliomyelitis-like syndrome with respiratory involvement is a condition associated with considerable death and illness; however, substantial and even dramatic recovery is sometimes observed in such patients. Persistent quadriplegia and respiratory failure lasting ≥4 months may indicate poor prognosis. Continued long-term assessment of patients recovering from WNV-associated paralysis is needed to fully discern the spectrum of possible outcomes.

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Human Pythiosis

To the Editor: Over the past 2 decades, human pythiosis has emerged as an important parafungal disease; Thailand reports the most cases (*1,2*). Given the rarity of this infection in humans and the limited attention of researchers to the disease, our understanding of its pathogenesis and other important traits, including its management, await investigation. We report 2 distinct cases.

The first patient was a 63-year-old woman with hemoglobin AEBart's disease, a complex thalassemia/hemoglobinopathy syndrome, which required frequent blood transfusions. Two months before admission, after a major flood and exposure to standing water for weeks, an abnormal sensation developed in her right foot, which progressed to pain and intermittent claudication. Subsequent inability to walk prompted her to seek medical assistance. At admission, she was febrile with absence of pulses on the right lower extremity and a diminished pulse on the left. She had a peripheral blood eosinophilia of 10%. Emergency femoral angiography indicated complete obstruction of the right common iliac and left internal iliac arteries (Figure) with collateral supplies via lumbar arteries. Surgical intervention demonstrated a white clot from aortic bifurcation down to the right common iliac artery and the superficial and deep femoral arteries, surrounded by necrotic tissue and enlarged inguinal nodes. Right femoral embolectomy and aorto-femoral bypass were performed. A 10% KOH preparation of the clot and dissected nodes showed branching septate hyphae.

Cultures of these specimens grew *Pythium insidiosum*. Itraconazole solution, terbinafine, and therapeutic vaccines (*3*) were administered. Extended-spectrum antimicrobial agents were also given during her hospital stay to combat nosocomial infections. Progressive wound necrosis and gangrene of the limbs dictated multiple debridements and subsequent limb amputations and hip disarticulation. Her clinical course worsened, and she died 2 months after admission.

The second patient was a 15-year-old boy with β-thalassemia/hemoglobin E disease, 10 years after splenectomy. The patient received frequent blood transfusions and monthly intravenous deferoxamine for secondary hemochromatosis. Six days before admission to our hospital, necrotizing cellulitis of both legs, unresponsive to intravenous antimicrobial agents, was diagnosed at a local hospital. The patient reported frequent swimming in standing water in rice fields. He was febrile with a leukocyte count of 26,000/mm^3 and was given broad-spectrum intravenous antimicrobial agents. Severity of the lesions demanded surgical debridement (online Appendix Figure, available from http://www.cdc.gov/ncidod/EID/vol12no03/05-1044-appG.htm), which showed necrosis of skin and subcutaneous tissue but intact fasciae and muscles. Tissue pathology and fungal staining did not demonstrate the etiologic agent. Surgical tissue Gram's stain and culture were nonrevealing, but fungal culture grew *P. insidiosum* after a few days. Isolates demonstrated white submerged colonies and microscopically showed sparsely septate hyphae 4–10 µm in diameter. Boiled grass blades were transferred onto the agar with the growth and incubated at 37°C for 24 hours. The blades were then put in a container with dilute salt solution for 2 hours at 35°C. They were subsequently placed on a slide, and mobile biflagellate zoospores were seen. Serum antibodies to *P. insidiosum* by immunodiffusion were detected in both cases. Femoral angiography showed no abnormalities. Since therapeutic immunogens were not available during that period, a combination of supersaturated potassium iodide (SSKI), itraconazole, and terbinafine was initiated. SSKI was discontinued after a month because of side effects. Lesions were progressive involving fasciae and muscles and necessitated

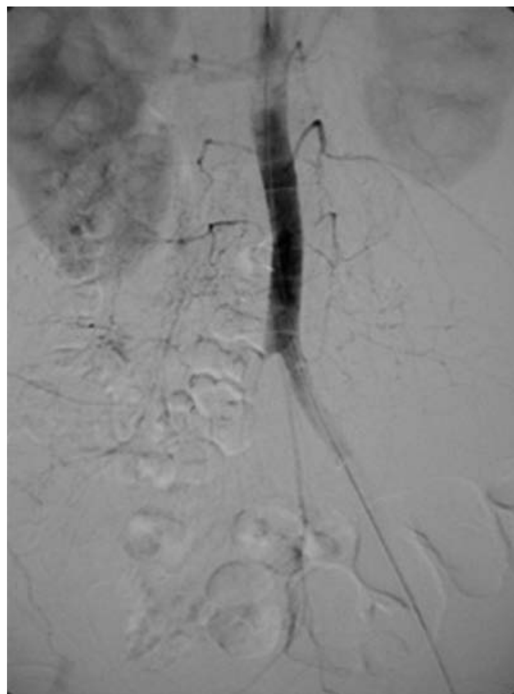

Figure. Femoral angiography of patient 1 demonstrating involvement of the right common iliac artery upward to the aorta and extending to the left internal iliac artery.

2 episodes of surgical debridement during the first month. The lesions began to improve in the second month of medical therapy, when skin grafting was performed. The patient remained well and was discharged after almost 3 months. Medications were continued for a total period of 6 months. The patient has been well for >2 years.

P. insidiosum in tissues resembles agents of zygomycosis morphologically but, unlike the latter, rarely stains with hematoxylin and eosin. Various immunostainings also help identify the organism (*4*). With the exception of the facial-cranial form of the disease in the United States (*5*), most cases in Thailand occur in patients with chronic hemolytic anemia; thalassemia-hemoglobinopathy is the most common underlying disease. Major clinical manifestations include ocular and craniofacial infections in healthy children, arteritis usually originating from lower extremities, and chronic subcutaneous abscesses.

Treatment options reported to be successful include supersaturated potassium iodide for the chronic cutaneous form (*2*), a combination of terbinafine and itraconazole in a single case of an acute, severe ocular, subcutaneous infection (*6*), and therapeutic vaccination for severe infections involving major arteries (*7,8*) in conjunction with surgery. The first case suggested that, with chronic pythiosis involving the aorta, effective management is difficult. In the second case, the laboratory was familiar with *P. insidiosum* isolation procedures; therefore, a quick diagnosis was made and early treatment was instituted. This early form of cutaneous pythiosis is rarely diagnosed properly by most clinical and pathologic laboratories. Human cases probably occur worldwide (*9*) but are underrecognized and thus, misdiagnosed (*5*). More research into the pathogenesis, diagnosis, and new treatment modalities is urgently needed.

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Rift Valley Fever Potential, Arabian Peninsula

To the Editor: Rift Valley fever (RVF) virus causes severe disease, abortion, and death in domestic animals (especially young sheep, cattle, and goats) in Africa and the Arabian Peninsula. Humans are infected by mosquitoes, which maintain epizootic transmission, or through exposure to infected animal tissue. Although human disease may be mild, sometimes severe retinitis, meningoencephalitis, or hemorrhagic fever syndromes may develop in patients. In Africa, epizootics and associated human epidemics usually follow heavy rainfall (*1*).

RVF was first confirmed outside Africa in September 2000. The outbreak in southwestern coastal Saudi Arabia and neighboring coastal areas of Yemen resulted in an epizootic with >120 human deaths and major losses in livestock populations from disease and slaughter (*2,3*). RVF virus isolated from the floodwater mosquito *Aedes vexans arabiensis* during the outbreak was closely related to strains from Madagascar (1991) and Kenya (1997), which suggests that the virus was imported through infected mosquitoes or livestock from East Africa (*3*). The Arabian outbreak followed

increased rainfall in nearby highlands that flooded the coastal areas and created ideal environments for mosquito populations similar to those found in RVF-endemic regions of East Africa (*4*). Most RVF activity was associated with flooded wadi agricultural systems; no cases were reported in the mountains or in dry sandy regions, where surface water does not accumulate long enough to sustain mosquito breeding.

To provide early warning of conditions favorable for RVF epidemics, the National Aeronautics and Space Administration (NASA) and the Department of Defense Global Emerging Infections Surveillance and Response System (DoD-GEIS) monitor the satellite-derived normalized difference vegetation index (NDVI), which reflects recent rainfall and other ecologic and climatic factors (*5–7*). NDVI anomalies in the highlands east of affected areas during the 2000 outbreak (online Appendix Figure panel A, available from http://www.cdc.gov/ncidod/EID/vol2no03/05-0973-G.htm) showed a spatial pattern (although of lower magnitude) similar to recent anomalies in those areas (online Appendix Figure panel B). Greater than normal NDVIs (20%–60%) were seen in the Sarawat Mountains, from just northeast of Djeddah, Saudi Arabia, and southwestward beyond Jizan and into Hodeidah governorate in Yemen during May and June 2005.

Satellite-derived rainfall estimates show that widespread rainfall occurred over most of western Saudi Arabia and Yemen from mid-April to mid-June 2005 (*8*) and accounts for the high magnitude and spatial pattern of observed NDVI anomalies in May and June 2005. Rainfall was concentrated in the mountainous regions east of the Red Sea coast, and was heaviest in the areas east of Djeddah and Jizan, with rainfall totals as high as 120 mm and 60–80 mm, respectively, during April 2005, compared with the same period in 2000 (10–50 mm) (online Appendix Figure panels C and D) and in southwestern Yemen, with totals as high as 120 mm during May. In the area east of Djeddah, total rainfall in April 2005 was 150 mm above the long-term average for that month. Flooding was reported in Hodeidah Governorate, Yemen during May (*9*) and could be expected in other Red Sea coastal areas following such heavy rainfall. This created habitats appropriate for breeding of mosquitoes capable of transmitting RVF, as occurred in 2000.

No human cases of RVF have been reported in Saudi Arabia and Yemen since the 2000 outbreak, but in September 2004 the Saudi Ministry of Agriculture reported that 5 RVF-seropositive sheep had been identified during routine surveillance in Jizan where most infected persons were exposed during the outbreak in 2000 (*2*). The primary infection was estimated to have occurred in April 2004 (*10*). The NDVIs and rainfall patterns alerted the Yemen and Saudi Arabia Ministries of Health and Ministries of Agriculture to conduct field investigations with the Food and Agriculture Organization and the World Health Organization.

Since RVF virus can be maintained in mosquito eggs for extended periods and transmitted under favorable conditions (*6*), the high magnitude of NDVI and rainfall patterns reported should prompt heightened veterinary and human surveillance for RVF in coastal Arabia and mass vaccination of susceptible animals. The current RVF model (*7*) is indicative of conditions that would promote vector breeding and could result in an outbreak of mosquitoborne diseases.

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Screening and Toxigenic Corynebacteria Spread

To the Editor: Diphtheria is rare in countries with high vaccination coverage, but as seen in Europe in recent decades, control can disintegrate rapidly. When diphtheria is rare, surveillance is challenging because clinicians have no experience with the infection, and disease may be mild or atypical in vaccinated persons (*1*). Clinicians may give inadequate information to laboratories, and appropriate investigations may not be performed. Identifying cases is facilitated if all throat swabs from patients with pharyngitis are screened by laboratories for corynebacteria, but this procedure is expensive and time consuming. To help balance priorities in diphtheria surveillance, we evaluated the potential benefits of microbiologic screening in preventing secondary spread of toxigenic corynebacteria in England and Wales and estimated the possible consequences of not detecting a case.

The mean number of secondary cases that might occur per index case if screening is not undertaken depends on the mean number of contacts and attack rates, vaccine coverage and efficacy, and duration of protection. Some of these factors are not known precisely, so we estimated them within plausible ranges of values. We varied the number of contacts per case-patient from 2 to 20. Secondary attack rates in susceptible persons are difficult to estimate and distinguish from carriage rates (*2*), and we varied these from 5% to 50%. Vaccine efficacy in children was varied from 50% to 95%. We estimated the susceptibility of UK adults at 40% (*3*), vaccination coverage in children at 95% (*4*), and case-fatality ratio at 6% to 10% (*5*). For simplicity, the ratio of adults to children among contacts was assumed to be 1:1. We assumed that without specific microbiologic identification of cases, no intervention would take place and that intervention to protect contacts is 100% effective. Such intervention includes early treatment and isolation of cases, chemoprophylaxis, and booster vaccination of contacts. The number of cases that need to be detected to prevent 1 secondary case for different numbers of contacts and attack rates was calculated as the inverse of the number of secondary cases that would result from each case not detected by screening.

The number of cases that must be detected by microbiologic screening to prevent 1 secondary case was most affected by varying the number of contacts per patient and the secondary attack rate (Figure). If one assumes vaccine efficacy of 95%, an attack rate in susceptible contacts of 5%, and 4 contacts per patient, 1 secondary case is prevented for every 18 cases detected; if attack rates are 30%, then 1 secondary case is prevented for <5 index cases detected. If vaccine efficacy was 50%, the number of cases that would need to be detected to prevent 1 secondary case would fall from 18 to <10 cases for a mean of 4 contacts per case and secondary attack rates of 5%.

For the 53 toxigenic strains of corynebacteria detected in England

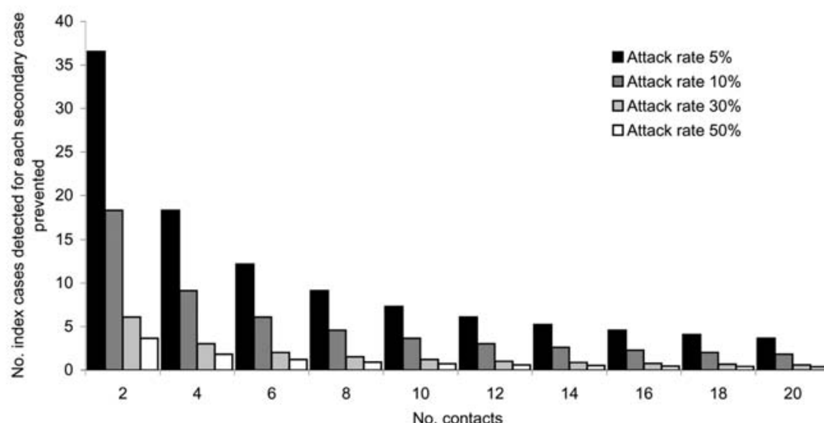

Figure. Number of cases needed to detect to prevent 1 secondary case.

and Wales from 1993 to 2000, an estimated 2–10 secondary cases would have been prevented if attack rates were 5% and each patient had 4 contacts. The number of index cases needed to be detected to prevent 1 death (assuming 6%–10% case-fatality ratio) would have been 150–180 with attack rates of 5% and 50–83 with attack rates of 30%. Thus, deaths were not likely to have been prevented during this period by screening.

Are the parameter estimates valid? We focused on secondary cases, but spread in outbreaks may be exponential, so the effect of missing cases may be greater once tertiary cases and further spread are taken into account. Vaccination coverage may be higher or lower in different risk groups. Secondary attack rates in the literature are reported from outbreaks and regions with vulnerable populations during periods of high incidence and may not apply in affluent countries with high coverage and may be <5%. Adult protection may be better than indicated by serosurveys and may have improved in the United Kingdom with use since 1994 of combined tetanus-diphtheria toxoid vaccine instead of tetanus toxoid for injuries (*5*).

Outbreaks are not reported from countries without routine screening (*1*), which indicates that some of our assumptions and estimates may be incorrect. Alternatively, this fact may indicate defective surveillance; countries that do not detect primary cases may not detect secondary cases.

Surveillance for diphtheria in European Union member states varies widely (*1*). Only 5 of 19 reporting countries screen throat swabs routinely for corynebacteria, raising doubts about the quality of surveillance. The absence of reports of diphtheria may not reflect the absence of disease or of circulating toxigenic corynebacteria. Our results show the possible consequences of not detecting such infections and help demonstrate the public health priority of diphtheria surveillance.

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Rickettsia slovaca Infection, France

To the Editor: *Rickettsia slovaca* was first isolated in 1968 in a *Dermacentor marginatus* tick collected in Slovakia, and serologic evidence of infection with this bacteria was reported in patients with enlarged lymph nodes and a scalp eschar after being bitten by a tick (*1*). However, the first proven case of *R. slovaca* infection was reported only in 1997 in France (*2*). This rickettsiosis is called tickborne lymphadenopathy (TIBOLA) because the most pronounced sign is lymph node enlargement. In Spain the same condition is called *Dermacentor*-borne-necrosis-erythema lymphadenopathy (*3,4*).

In this study, we describe 14 new patients with TIBOLA from southern France who sought treatment from January 2004 to May 2005 and compare the features of these patients with those in whom Mediterranean spotted fever (MSF) was diagnosed during the same period. All the patients were referred to our center with a suspected rickettsial infection characterized by a tick bite located on the scalp, an inoculation eschar, and enlarged lymph nodes (see online Appendix Figure, available at http://www.cdc.gov/ncidod/EID/vol12no03/05-0911-appG.htm). For each patient, an acute-phase and a convalescent-phase serum sample were obtained for serologic analysis. Culture and polymerase chain reaction (PCR) were performed on tick, skin biopsy, or blood specimens. A multiple-antigen immunofluorescence assay (IFA) was performed by using 5 spotted fever group (SFG) rickettsial antigens: *R. conorii conorii*, *R. slovaca*, *R. helvetica*, *R. sibirica mongolitimonae*, and *R. felis*. Titers of at least 64 for immunoglobulin G (IgG) and 32 for IgM in acute-phase serum samples, evidence of seroconversion with 4-fold increases in IgG titers, or both, were considered as evidence of recent

infections with a *Rickettsia* sp. (*5*). For serum specimens confirmed by IFA at the species level, Western immunoblotting and cross-adsorption assays procedures were performed as described elsewhere (*6*) by using *R. conorii conorii* and *R. slovaca* antigens. Patients with a definite serologic diagnosis at the species level were analyzed for their epidemiologic and clinical information.

Culture from skin biopsy specimen and ticks were injected into human embryonic lung cells and cultivated into shell-vial culture as previously described (*7*). DNA was extracted from skin biopsy specimens, acute-phase serum samples, and ticks by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) (*8*). Standard PCR was performed with primers suitable for hybridization within the conserved region of genes coding for outer membrane protein A (*ompA*) and citrate synthase (*gltA*) (*8*).

Among the 14 patients in a scalp lesion and cervical or occipital (1 case) lymph node enlargement developed after they were bitten by a tick, 9 were female (1 was pregnant) and 5 were male. The median (range) age was 34.9 (5–85) years with half of the patients <10 years of age. The incubation period ranged from 5 to 15 days (median 10.5 days; n = 7). Only 3 patients had fever. All patients fully recovered with doxycycline or, for the pregnant patient, josamycin therapy. Serology confirmed the diagnosis of *R. slovaca* infection for 10 patients by microimmunofluorescence and Western blot analysis after cross-adsorption studies (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no03/05-0911.htm#apptable). *R. slovaca* was amplified by PCR for 7 cases, including 3 skin biopsy specimens, 3 *Dermacentor marginatus* ticks, and 1 acute-phase serum sample (Appendix Table). Three isolates (2 from skin biopsy specimens and 1 from a tick) were obtained by using the shell-vial culture assay. During the period of our study, in the same French region, 40 patients with MSF were clinically and laboratory diagnosed using the same procedures. The median (range) age was 54.2 (5–85) years with only 3 children <10 years of age (compared to 7/7 children with *R. slovaca* infection, p = 0.0015). MSF occurred mainly during the summer, whereas *R. slovaca* infection was seen during the colder months with 6 cases from October to January and 8 cases from February to May (Figure).

In France, *R. conorii* has long been considered to be the only SFG rickettsiosis but *R. slovaca* may also be prevalent (*9*), contributing 25% of the cases in the present study. This organism is also a common cause of disease in Hungary and in La Rioja, Spain (*3*). These data suggest that TIBOLA mainly occurs in young children, affects women predominately, and occurs primarily during the colder months (*9,10*). As previously reported (*9*), we found that standard microimmunofluorescence serologic testing was insensitive and that Western blot is more useful and allows identification to the species level after cross-adsorption studies. Finally, DNA amplification by PCR from skin biopsy tissue, serum samples, or in ticks allowed confirmation of the diagnosis in only 50% of the cases, which suggests that other rickettsial species may be responsible for TIBOLA. Epidemiologic and clinical presentations are so characteristic that the clinical diagnosis should be considered in patients who have been bitten on the scalp during the colder months. In Europe, *R. slovaca* infection is likely to be a significant cause of cervical lymph node enlargement, and microbiologic investigation and tick analysis will underline the relative importance of this disease.

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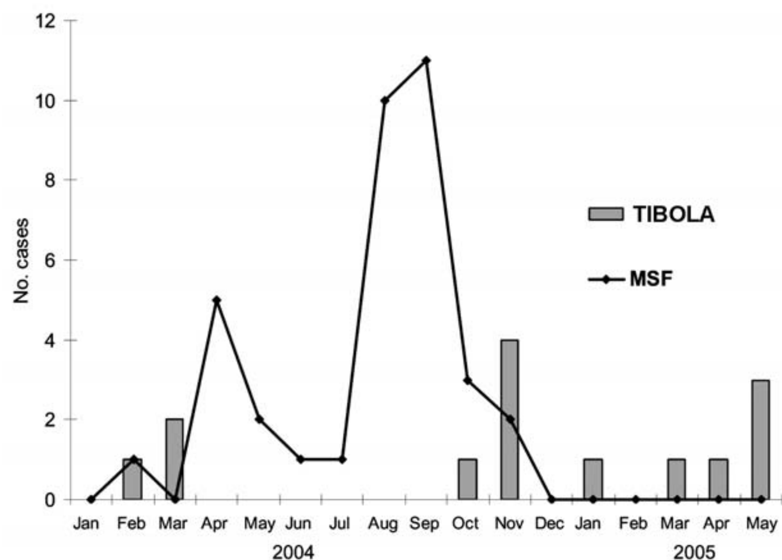

Figure. Seasonal distribution of Mediterranean spotted fever (MSF) and tickborne lymphadenopathy (TIBOLA) in southern France from January 2004 to May 2005.

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Cutaneous Anthrax, Belgian Traveler

To the Editor: Anthrax is a rare zoonotic disease among travelers. The clinical spectrum includes cutaneous lesions, respiratory anthrax, pharyngeal inflammation, gastrointestinal infection, septicemia, and meningitis. Interest in anthrax increased after the bioterrorist attacks in the United States in 2001. The following case history describes a cutaneous infection suspected to be anthrax in a tourist who had indirect contact with dead mammals in a disease-endemic area.

After indirect contact with dead antelopes and a hippopotamus in Botswana, an acute necrotic lesion developed on a finger of a 31-year-old, healthy, female Belgian woman. The lesion became covered with a black crust, followed by massive swelling of the hand and arm. The clinical aspect and history strongly suggested cutaneous anthrax. This diagnosis was supported by seroconversion to protective antigen of *Bacillus anthracis* and the presence of antibodies against lethal factor. The bacterium itself could not be cultured or identified by polymerase chain reaction (PCR). Other members of the group with which she traveled were contacted, but no other cases were reported.

The Belgian woman traveled with friends to Namibia, Botswana, and South Africa from December 12, 2004, until January 22, 2005. She visited Chobe National Park in Botswana early January 2005. On January 8, a small, painless, vesicular lesion developed on the dorsal side of her fourth left finger. This lesion increased in size quickly and developed a black aspect with a red elevated border. Small vesicles appeared in the immediate vicinity of the primary lesion. No pus was noted. Her general condition was good. She treated herself with amoxicillin-clavulanic acid 2 gm/day for 3 days. The next day, massive edema of the finger, hand, and left arm developed. When admitted to a hospital in Johannesburg, her left arm and hand were massively swollen with painful left axillary lymphadenopathy. Her temperature never exceeded 37.8°C. Wound cultures showed only the presence of viridans streptococci, bacteria that are not implicated in wound infections. The patient was treated with intravenous ciprofloxacin, gentamicin, tetracycline, flucloxacillin, and topical mupirocin. She was discharged after 6 days with oral flucloxacillin and returned to Belgium on January 22. On February 4, her general condition was excellent; the edema had diminished. A painless necrotic lesion on the left fourth finger measured 3 cm^2 (Figure). She mentioned minor discomfort of her left underarm and loss of sensation at the distal radial side of the left underarm. She could not extend the terminal phalanx of the fourth left finger because the underlying tendon had been destroyed. The left axillary lymph nodes were still slightly swollen. No evidence indicated parapox viral infection or necrotic arachnidism. Upon questioning, she mentioned that in Chobe National Park, some fellow travelers had manipulated the legs of dead antelopes. One person had climbed on a dead hippo for a picture and sank into the putrefying carcass. He soon afterwards cleaned a small abrasion on the patient's finger. Some hours later, all group members washed their hands in a common small plastic basin containing water and chloroxylenol.

Full blood count, erythrocyte sedimentation rate, and biochemistry were normal. Antistreptolysin O levels were within normal limits. Serologic test results for rickettsiae, orthopoxviruses, and *Bartonella henselae* were negative. The patient was not

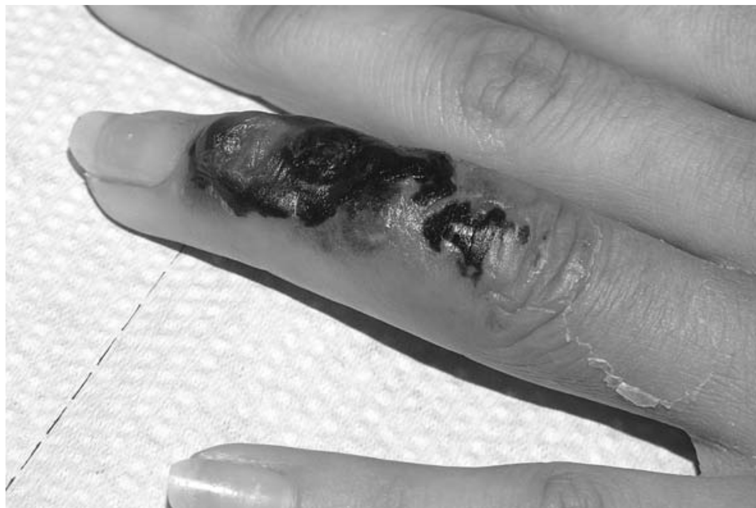

Figure. Initial skin lesion, suggestive of cutaneous anthrax. By the time the picture was taken, the massive edema of hand and arm had subsided.

immunocompromised. Because cutaneous anthrax was suspected, wound crusts, swabs for bacterial cultures, and Dacron swabs used for PCR were mailed as quickly as possible to the Belgian national reference laboratory. All cultures remained sterile. PCR was negative for *B. anthracis.* Because of the positive clinical outcome with antimicrobial drugs for 16 days, no additional antimicrobial drugs or steroids were prescribed. Further recovery was uneventful and only a small scar remains. While waiting for serologic test results, a ProMed alert was issued (*1*). Members of the travel group were contacted and warned but no other cases were identified. Consecutive serum samples were analyzed for *B. anthracis* protective antigen antibodies (anti-PA) (Centers for Disease Control and Prevention, Atlanta, GA, USA). The serum collected on February 4 was negative. On February 16, anti-PA immunoglobulin G (IgG) was detected with a titer of 9.5 (weakly positive). On April 18, no anti-PA IgG could be detected. Paired serum samples (February 4 and 16) were also mailed to the Institut für Microbiologie der Bundeswehr in Munich, Germany. In the German laboratory, the anti-PA enzyme-linked immunosorbent assay result was negative, but specific antibodies against lethal factor of *B. anthracis* were detected.

Anthrax is essentially a disease of grazing animals and is relatively common in persons who have contact with these animals (*2–4*). It is occasionally reported in travelers (*5*). In this case, many arguments existed for cutaneous anthrax, but the diagnosis could not be proven. Clinical symptoms (malignant edema) and history of indirect contact with carcasses of wildlife in a disease-endemic area suggested anthrax. Bacterial cultures remained negative, presumably because of previous administration of antimicrobial drugs. The clinical diagnosis was supported by seroconversion to protective antigen and the presence of antibodies against lethal factor. In cutaneous anthrax, antibodies to protective antigen develop in 68%–92% of cases (*6,7*). Previous cases of cutaneous anthrax in Belgium date from the 1980s, when a man became infected while unloading Indian bone meal in Antwerp Harbor. In 1986, cutaneous anthrax developed in a Turkish woman after being injured while cooking a sheep (*8*). In 2002, a suspected case in a Belgian farmer was reported (*9*). Many cases of cutaneous anthrax heal spontaneously, but a 5%–10% chance of systemic complications exists. This case illustrates 1 of the dangers of touching dead animals in nature. Travelers should be warned that even indirect contact can lead to problems.

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Japanese Encephalitis, Singapore

To the Editor: Japanese encephalitis (JE) is an endemic flavivirus disease in Asia. The JE virus (JEV) is one of the leading causes of viral encephalitis: 35,000–50,000 cases occur every year (*1*). While most infections are subclinical, the disease has a high case-fatality rate (≈25%) and considerable incidence of serious neurologic sequelae with the development of overt meningoencephalitis (*1*).

JEV is transmitted principally by *Culex tritaeniorhynchus* and less frequently by *Cx. vishnui* and *Cx. gelidus*, which breed in flooded rice fields. The virus circulates in waterfowl such as herons and egrets, and pigs serve as amplifying hosts. Hence, the distribution of JEV is significantly linked to irrigated rice production and pig rearing (*2*).

JEV was previously endemic in Singapore, but since the phasing out of pig farming (completed in 1992), the incidence of reported disease has become very low. Routine serologic testing for JEV has correspondingly been dropped from local hospital microbiology laboratories. We describe an indigenous case of JEV meningoencephalitis in Singapore.

In May 2005, a 53-year-old previously healthy man of Chinese ethnicity was seen at Singapore General Hospital with a 1-week history of fever and abdominal pain. Altered mental status had developed shortly after the onset of fever. He had worked in the western part of Singapore as a lifeguard at a community swimming pool and had not traveled, even to offshore islands, for the past year.

On examination, he was febrile with a temperature of 39.3°C and disoriented to time and place. Nuchal rigidity was present, and hyperreflexia was demonstrated in both upper limbs, although lower limb reflexes were normal. The rest of the initial physical examination was unremarkable.

Laboratory studies showed a leukocyte count of 4.91×10^9/L, hemoglobin concentration of 14.3 g/dL, and platelet count of 171×10^9/L. Serum and liver biochemistry results were normal. Magnetic resonance imaging of the brain showed mild leptomeningeal enhancement. An electroencephalogram showed generalized slow waves, consistent with severe diffuse encephalopathy. A lumbar puncture was performed. The opening pressure was elevated at 24 cm/H_2O; cerebrospinal fluid (CSF) leukocyte count was 192/mm^3, consisting mostly of lymphocytes; CSF glucose was 2.4 mmol/L (44% of serum glucose concentration); and CSF total protein was elevated at 1.5 g/L. CSF and blood cultures for bacteria, fungi, and mycobacteria were negative, as were CSF isolates for enteroviruses and herpes simplex virus.

Results of paired acute- and convalescent-phase serologic testing for dengue immunoglobulin M (IgM) and IgG were negative, as were the microscopic agglutination test for leptospirosis and the Widal test for typhoid. Subsequent polymerase chain reaction (PCR) testing of serum and CSF on day 10 of illness yielded negative results for Nipah/Hendra virus, West Nile virus, enterovirus, herpesviruses, measles virus, and alphaviruses.

However, the patient's serum but not CSF tested positive for flavivirus RNA when a universal flavivirus reverse transcription (RT)–PCR assay that targets the conserved sequence of the NS5 region was used (*3*). JEV was definitively identified as the etiologic agent when the serum sample tested positive with a second RT-PCR specific to the conserved sequences in the NS3 region of the JEV genome, modified to a real-time platform (*4*). Comparison of the 197-nt sequence of this JEV-specific RT-PCR product with the library of human, mouse, and viral genome databases managed by the National Center for Biotechnology Information site using the BLASTN program (available from http://www.abcc.ncifcrf.gov/app/htdocs/appdb/appinfo.php?appname) showed 93% homology with reported JEV sequences.

The patient had a prolonged and complicated hospital stay. He became comatose and went into type 2 respiratory failure within 72 hours of hospitalization; pinpoint pupils, bradycardia, and hypothermia developed. These developments necessitated mechanical ventilation at the medical intensive care unit, where the patient subsequently improved after 6 days of supportive care and was extubated. Flaccid paraparesis with urinary retention developed at this point, and magnetic resonance imaging of the spine demonstrated signal enhancement at the level of the conus medullaris. Motor power gradually improved with intensive rehabilitation and was normal by the time of the patient's discharge 2 months after admission. However, intermittent self-catheterization was still required for detrusor hyperreflexia.

This is the sixth case of JE reported in Singapore from 1991 to July 2005. Three imported cases were reported from 1991 to 2000. Two

patients whose cases were reported in 2001 had no substantial travel history and likely acquired the infection within Singapore, as our patient did. However, the lack of diagnostic testing by local service microbiology laboratories has possibly led to underdiagnoses of this disease.

While abolishment of pig farming in Singapore has greatly reduced the risk for epidemic transmission of JEV, a seroepidemiologic study on the prevalence of neutralizing antibodies to JEV in local animals (including dogs, cattle, goats, imported pigs, chickens, and crows) showed a JEV antibody prevalence of 46.5% in working dogs and 60% in chickens. These findings suggest that JEV remains active in Singapore (*5*). The virus reservoir is likely to be aquatic birds. The threat of JE remains, and public health vigilance for this vectorborne disease should not diminish.

The infrequent incidence of JE in Singapore is insufficient to justify routine vaccination for travelers to this country. However, JE remains a rare differential diagnosis for travelers from or passing through Singapore.

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HIV and Lacaziosis, Brazil

To the Editor: Jorge Lobo disease (lacaziosis) is a chronic deep mycosis for which prognosis is good in terms of survival but unclear in terms of regression of the lesions (*1*). No involvement of internal organs or mucous membranes is observed. The causative agent is *Lacazia loboi* (*2*), a fungus of uncertain phylogeny, which causes an inflammatory infiltrate accompanied by the formation of a granuloma in which giant cells phagocytose a larger number of fungi (*3,4*). Pecher and Funchs suggested that patients with lacaziosis have a cellular immunodeficiency (*5*). The disease is more frequent in men and persons 21–40 years of age. It is found exclusively in Latin America; only 1 case has been diagnosed in Europe, and that was due to accidental contamination with material from a dolphin (*4*).

Trauma and injuries or sites of insect bites facilitate penetration of the fungus. Lesion progression is slow, with new lesions arising by contiguity with other lesions or through the lymphatic route (*6,7*). Clinically, lacaziosis manifests as keloidal lesions of solid consistency and variable size that contain small scales and crusts (*6*). The lesions are most frequently located in the auricle and on the upper and lower limbs. Cutaneous dissemination of the disease is observed in a relatively small number of cases. We describe a patient with Jorge Lobo disease.

The patient was a 59-year-old man, a storeroom employee, who was seen at the Tropical Medical Center in Belem, Brazil, in April 2004. A papula had developed near his right knee in 1992 after a wood splinter had penetrated the skin. The lesion increased in size, and a histopathologic diagnosis of Jorge Lobo disease was made. The lesion was then surgically removed. Approximately 2 years later, the lesion recurred. The patient then went to a dermatology service and was treated with clofazimine, after which the lesion disappeared. However, the lesion reappeared 1 year later.

HIV serologic analysis was performed in 2002, and the results were positive. The patient then began treatment for HIV infection. He is currently being monitored at the specialized referral unit in Belem. He does not have any opportunistic infections and is not taking any antiretroviral drugs. The patient came to the dermatology service of the Tropical Medical Center, where dermatologic and histopathologic examinations were conducted and CD cell counts and HIV viral load were measured. Dermatologic aspects of the lesion included an erythematous-infiltrated, hypertrophic plaque with a verrucous surface ≈4 cm long in the distal third of the medial aspect of the right thigh (Figure). A punch biopsy specimen of brown smooth skin 0.35 cm in diameter in an epidermal disk was fixed in formalin. Microscopy of skin sections containing epidermis showed compact keratinization, parakeratotic foci,

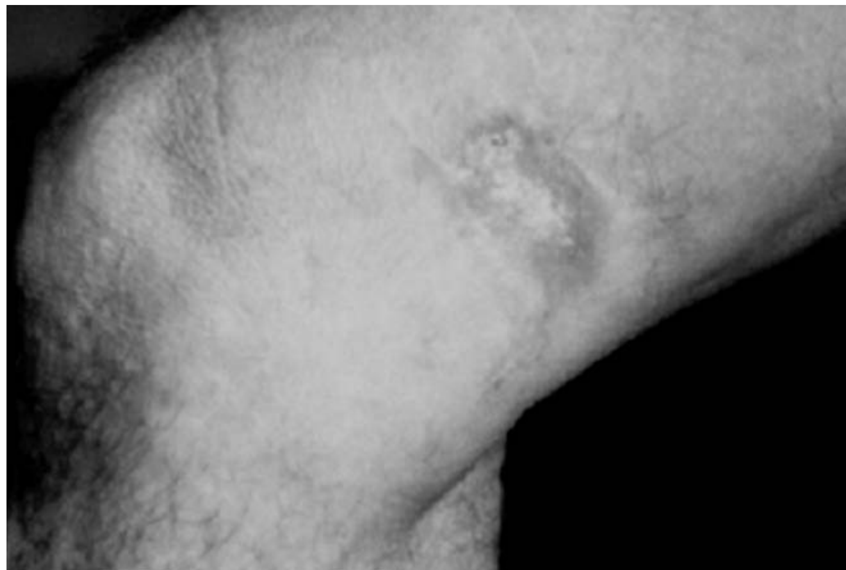

Figure. Erythematous-infiltrated, hypertrophic plaque with a verrucous surface ≈4 cm long in the distal third of the medial aspect of the right thigh of the patient.

and irregular hyperplasia with a pseudoepitheliomatous area. A highly dense, nodular, diffuse inflammatory infiltrate was observed at all levels of the dermis. It consisted of macrophages and numerous multinucleated cells, most of them of the foreign body type. Fibroplasia was also noted. Abundant, round parasitic elements surrounded by a double membrane and containing a basophilic nucleus were found in tissues, as well as other anucleated, intracellular, and free parasites that formed chains of ≥2 cells (online Appendix Figure, available from http://www.cdc.gov/ncidod/EID/vol12no03/05-1426_appG. htm). Jorge Lobo disease was diagnosed. Laboratory results showed 146 CD4 cells/µL, 251 CD8 cells/µL, a CD4:CD8 ratio of 0.42, and 60,000 copies of HIV viral RNA/mL.

Since a cytotoxic response is observed in Jorge Lobo disease (*7*), HIV infection may increase the susceptibility to infection with *L. loboi*. Patients with AIDS show a predisposition to diverse fungal infections that classically affect different organs and systems. An association between Jorge Lobo disease and AIDS has not been reported. However, since Jorge Lobo disease is restricted to specific areas of the world and the number of AIDS cases is increasing, especially in Latin America, a possible correlation between HIV infection and Jorge Lobo disease should be considered because of the associated cellular immunodeficiency.

The patient showed no signs of other opportunistic infections classically associated with AIDS, and he was not taking any antiretroviral drugs. His initial infection manifested as cutaneous lesions that occur in Jorge Lobo disease. Despite the cellular immunodeficiency, we did not observe atypical dissemination of the lesions. Further studies should be conducted to evaluate the relationship between the cellular immunosuppression of AIDS and secondary infection with *L. loboi*. In addition, epidemiologic studies are needed to determine the association of AIDS with Jorge Lobo disease.

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Hand Sanitizer Alert

To the Editor: Community-based epidemiologic studies have shown beneficial effects of hand sanitizers. Hand sanitizers were effective in reducing gastrointestinal illnesses in households (*1*), in curbing absentee rates in elementary schools (*2*), and in reducing illnesses in university dormitories (*3*). An Internet search retrieved recommendations for hand hygiene from schools, daycare centers, outdoor guides, and animal shelters.

To reduce infections in healthcare settings, alcohol-based hand sanitizers are recommended as a component

of hand hygiene (*4*). For alcohol-based hand sanitizers, the Food and Drug Administration (FDA) (*5*) recommends a concentration of 60% to 95% ethanol or isopropanol, the concentration range of greatest germicidal efficacy. While nonhealthcare groups also recommend alcohol-based hand sanitizers, they usually do not specify an appropriate concentration of alcohol.

Some products marketed to the public as antimicrobial hand sanitizers are not effective in reducing bacterial counts on hands. In the course of a classroom demonstration of the comparative efficacy of hospital-grade antimicrobial soap and alcohol-based sanitizers, a product with 40% ethanol as the active ingredient was purchased at a retail discount store. Despite a label claim of reducing "germs and harmful bacteria" by 99.9%, we observed an apparent increase in the concentration of bacteria in handprints impressed on agar plates after cleansing. None of the other hand cleaners showed such an effect.

Subsequently, we conducted more formal handwashing trials to verify the preliminary finding. Our goal was not to test the products by using the FDA tentative final monograph standard (*5*) but to determine whether a marketed product fails as an antiseptic because of its low alcohol content. To test whether the relatively low concentration of ethanol was the source of treatment failure, we included trials with laboratory-formulated 40% ethanol; we also supplemented the suspect gel with ethanol to a final concentration of 62%. Five hand hygiene treatments were compared: tap water (4 trials), 40% ethanol (5 trials), commercial gels with active ingredients of either 40% or 62% ethanol (9 trials each), and commercial 40% gel supplemented to 62% (5 trials).

At the beginning of each work day, the dominant hand of each volunteer was placed on 150-mm tryptic soy agar plates for 5 s, followed by hand treatment. Each alcohol-based hand treatment involved wetting the hands with 1.5 mL test product followed by vigorously rubbing hands together for 15 s. The tap water treatment differed in that hands were held under running water and vigorously rubbed together for 15 s, followed by air drying. After hands were dry, they were reapplied to a fresh plate for 5 s. Participants were assigned to treatments randomly, but each had to complete each treatment in a week. CFU counts before and after treatment were log transformed to normalize data and compared by using paired *t* tests.

Tap water, 40% ethanol, and 40% ethanol gel yielded no significant reductions in CFU (Table). The 40% gel supplemented with ethanol to a final concentration of 62% reduced the mean CFU by 90%, a level of reduction similar to that of the 62% ethanol gel. Moreover, the 62% gel and the supplemented 40% gel reduced CFU by >50% on all participants. In contrast, only one third of participants showed >50% reductions with 40% gel, one fifth with 40% ethanol, and none with tap water. Differences in pretreatment CFU were not significant (analysis of variance $F = 1.81$, df = 4, 27, $p = 0.16$). In addition to failing to decrease CFU, colonies were more evenly distributed on postwash plates after use of 40% gel. The even postwash colony distribution may be caused by dispersion of aggregates of microbes without sufficient killing.

Qualitative colony assessment suggested 40% gel and 40% ethanol were as effective as 62% gel against fungi; in contrast, bacterial CFU tended to show little change or increases. The most prevalent bacteria were staphylococci, including those with characteristics of *Staphylococcus aureus*.

After conducting experiments, a survey of 6 local retail chains found no substandard products. In the fall of 2005, a more extensive survey of 18 retail chains (supermarkets, drug stores, general retailers, specialty shops) uncovered a substandard product at all 3 stores of 1 deep-discount chain. The marketing profile of deep-discount chains suggests that poorer segments of the population may be more at risk of purchasing inadequate antiseptic gels. Moreover, 40% ethanol products may be stockpiled in homes and offices. An extensive Internet survey identified no additional substandard commercial products. However, the alcohol content of less-common brands was not always available online, and several Internet sites provide recipes for a bubble gum–scented children's hand sanitizer that contains 33% isopropanol as the sole active ingredient. Educational efforts should emphasize that effective sanitizers must be of a sufficient alcohol concentration.

The efficacy experiments reported here reinforce what has been known for >50 years: 40% ethanol is a less effective bacterial antiseptic than 60% ethanol (*6*). Consumers should be

Table. CFU per plate before and after treatment with various concentrations of ethanol

Treatment	Mean pretreatment CFU (range)	Mean posttreatment CFU (range)	No. trials	|*t*|*	p*	Mean change (%)
Tap water	175 (117–234)	206 (100–321)	4	1.25	0.30	+10
40% ethanol	531 (132–1,413)	621 (75–1,733)	5	0.30	0.39	+3
40% gel	246 (51–602)	232 (56–693)	9	0.61	0.56	+53
62% gel	171 (33–563)	12 (1–24)	9	5.73	<0.001	–82
40%→62% gel	473 (114–1,257)	26 (10–48)	5	6.21	0.003	–90

*|*t*| = result of paired *t* test; p = probability of |*t*|.

alerted to check the alcohol concentration in hand sanitizers because substandard products may be marketed to the public.

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Spleen Abscess as Malaria Complication

To the Editor: Changes in spleen structure, frequently encountered during malaria, may result either in a simple asymptomatic enlargement or in serious complications such as hematoma, rupture, or infarction (*1–3*). Hematoma or infarction of the spleen might be followed by the development of a splenic abscess, a clinical condition that has been reported in only 1 patient, to our knowledge (*4*).

A 21-year-old woman sought treatment at the hospital outpatient department of "Emergency," an Italian nongovernmental organization (NGO) in Freetown, Sierra Leone in May 2004, reporting malaise and persisting dull abdominal pain, accompanied by isolated episodes of spiking fever. Several recurrent malaria attacks (*Plasmodium falciparum*) had been reported by this patient in the last 2 months. At physical examination, conjunctival pallor and a tender, enormously distended abdomen were observed. A large abdominal mass, extending from the xiphoid process to the pubis, was palpable. Lymph nodes (neck, axillary, inguinal) were normal. Laboratory features showed severe anemia (hemoglobin 62 g/L; hematocrit 0.24), with low platelet count ($90 \times 10^3/\mu L$) and elevated leukocyte count ($130 \times 10^3/\mu L$), together with a moderate increase in liver enzymes (both aspartate aminotransferase and alanine aminotransferase were more than twice the upper limit of normal values). No malaria parasites were observed on blood smear at admission. Results of an HIV test were negative as were results of a sickle cell test, and hemoglobin electrophoresis results were normal. Other evident septic foci (e.g., typhoid fever, urinary tract infection, osteomyelitis) were excluded. Stool and urine examination excluded schistosomiasis. Blood cultures were not available.

An abdominal radiograph showed intraperitoneal fluid without distension of the bowel, whereas results of an abdominal ultrasound, performed in a private laboratory, diagnosed a large tumor on the left ovary. After receiving a blood transfusion (2 units) and intravenous antimicrobial drug treatment (ampicillin 500 mg 4 times/day, chloramphenicol 1 g 2 times/day, and metronidazole 500 mg, 2 times/day), the patient was scheduled for an exploratory laparotomy. Abdominal paracentesis was performed the day before surgery, and 2 L of thick brownish fluid was extracted.

An explorative laparotomy found ≈3 L of infected fluid in the peritoneal cavity. Widespread fibrin membranes covered thickened ileal loops. The mass was found to consist almost entirely a very large abscess on the spleen (Figure), which contained ≈5 L of pus. Dense adhesions were observed between the spleen, greater omentum, liver, and ileal loops. The liver was normal, and portal hypertension was not found. After splenectomy, the spleen's length was found to be 48 cm, and its weight was 6 kg. On histologic examination, splenic tissue was found to have been replaced by congested inflammatory infiltrates and fibrotic tissue. Leishmaniasis was excluded at microscopic examination. The patient completely recovered after surgery.

An enlarged spleen is found in 50% to 80% of malaria patients (*1*),

Figure. Dimensions of the abscess cavity are shown during the operation.

while only 25 cases of splenic rupture have been reported since 1960 (0%–2% in natural occurring infection) (*5*). A break of a contained hematoma is usually involved in splenic rupture, which occurs almost exclusively during acute infection and the primary attack (*6*). The incidence of splenic hematoma without rupture is unknown (*2*).

Spleen infarction is rarer than rupture and may go unnoticed. Only 9 documented cases of splenic infarction associated with malaria have been reported (*3*), all consequent to *P. falciparum* infection (except in 1 patient who was coinfected with *P. vivax* and 2 cases in which the etiologic agent was unknown). Splenic rupture following infarction has not yet been described.

Recently, an abscess of the spleen caused by *Salmonella enterica* serovar Enteritidis has been reported as a complication of *P. falciparum* malaria (*4*) and, to our knowledge, is the only case in the literature definitely related to *Plasmodium* infection. Indeed, splenic hematoma or infarction, together with the humoral and cellular immunodepression due to malaria, might well be predisposing factors for bacterial (e.g., salmonellae) colonization of the spleen from the gut, as likely happened in this patient, although cultures of the pus, blood, or intraabdominal fluid were not performed. Bacteremia caused by nontyphoidal salmonellae was significantly associated with malaria parasitemia (*7*), and splenic abscess has been recently reported as an atypical presentation of salmonellosis (*8*). Splenic abscesses caused by *Salmonella* infection usually occur on preexisting lesions (*4*) and have been increasingly reported recently (*9*).

Because of its insidious symptoms, a spleen abscess remains a diagnostic challenge in developing countries, where ultrasounds and computed tomographic scans are not easily accessible. Moreover, as in our patient, a spleen abscess is unlikely to develop as an immediate complication of malaria.

While splenectomy was the only possible treatment in this patient, a conservative approach, whenever possible, is always desirable, especially in the tropics, where the exposure to infective agents is particularly widespread. The overall prognosis of splenic abscesses remains discouraging, with 13%–16% of cases resulting in death (*9*), mainly consequent to late diagnosis and admission to a hospital. The growing volume of international travel will likely lead to an increase in the incidence of splenic complications in malaria patients, even in areas where the disease is not endemic. Therefore, clinicians should always keep the possibility of a superimposed abscess in mind.

Acknowledgment

We are deeply indebted to the medical and nonmedical personnel of the “Emergency” hospital in Goderich-Freetown, as well to the Italian NGO “Emergency” for their participation in this study.

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Rickettsioses in South Korea, Materials and Methods

To the Editor: We read with interest the article by Choi et al. (*1*), which describes the molecular detection of *Rickettsia typhi* and 4 spotted fever group rickettsiae by nested polymerase chain reaction (PCR) in the serum of febrile Korean patients. The value of the study, however, is limited by imprecision, inconsistencies, and the impossibility of verifying data. First, neither epidemiologic nor clinical data are provided for studied patients, although these are essential for interpreting PCR results. Second, multiplex nested PCR is hampered by a high risk of contamination (*2*). Alternatively, nested PCR techniques that use a closed assay or single-use primers without positive controls limit such a risk (*3*). In all cases, the use of negative controls is critical (*2,3*). In this study, negative controls are neither described in the Materials and Methods section nor shown on the gels. In addition, the authors used as positive controls 4 of the 5 *Rickettsia* species they detected. Therefore, apart from *R. felis*, which was not used as a positive control, positive products may result from cross-contamination. Finally, technically, the data are impossible to reproduce: 1) primer sets WJ77/80 and WJ79/83/78 cited in the legends of Figures 2 and 3 are neither described nor referenced in the text, 2) sequence of the RpCS.877p primer in Table 1 differs from that in the referenced article (*4*), 3) described sequences have not been deposited in GenBank, and 4) all *rompB* primers described in Table 1 exhibit 1–6 nucleotide mismatches with *ompB* sequences of at least 1 of the detected species. Based on these errors, the 7 cases of dual infections with *R. conorii* and *R. typhi*, which have never been reported before, are doubtful, and these data need to be confirmed.

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Rickettsioses in South Korea, Data Analysis

To the Editor: Choi et al. (*1*) conducted a study on sequence analysis of a partial *rompB* gene amplified from sera of humans who were seropositive for spotted fever group (SFG) and typhus group rickettsioses. They write, "These finding suggested that several kinds of rickettsial diseases, including boutonneuse fever, rickettsialpox, *R. felis* infection, and Japanese spotted fever… are occurring in Korea."

These claims propagate some errors and may lead to an inadequate conclusion. First, *rompB* is conserved in *Rickettsia* spp. and consists of 4,968 bp with respect to the published sequence of the *R. conorii* strain Seven (*2,3*). Fournier et al. (*4*) amplified 4,682 bp of *rompB* and showed a high degree of nucleotide sequence similarity (99.2%) between *R. africae* and *R. sibirica*, *R. pakeri*, and *R. slovaca*. Choi et al. amplified ≈420 bp of *rompB* (position 3562–4077) for sequence analysis. This segment is located in a highly conserved region of the gene, which may decrease the ability to differentiate particular species from other SFG rickettsiae. This study cannot prove the existence of specific SFG rickettsioses until the results are confirmed further by, for example, isolating these SFG rickettsiae from humans, animals, or ticks in South Korea. Recently, the authors analyzed nucleotide sequences of 267-bp amplicons of *rompB* (position 4762–4496) obtained from patient sera and found that *R. conorii* could not be differentiated from *R. sibirica* (*5*). This finding also supports our concerns.

Second, although partial *rompB* nucleotide sequence analysis of rickettsiae obtained from 1 patient's serum showed 98.87% similarity with *R. conorii* strain Seven, the finding does not indicate boutonneuse fever is

occurring in South Korea because high similarities (98.6%–99.8%) are found among 4 subspecies of *R. conorii*. Multilocus sequence typing can help differentiate among these subspecies (*6*).

This study provided a model to amplify SFG rickettsial DNA from sera of patients, and it will be helpful in surveillance of these diseases. However, the results should be interpreted more carefully in the context of clinical and epidemiologic data and combined with different gene sequence analyses to obtain a reliable and specific diagnosis.

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"Mycobacterium tilburgii" Infections

To the Editor: Advanced molecular biologic methods have improved the species differentiation and taxonomic classification of microorganisms, including nontuberculous mycobacteria. Identifying and characterizing an increasing number of "new" mycobacterial species of medical importance is now possible. Often, these newly described mycobacteria have been isolated from immunocompromised patients (*1,2*). Some of those have been difficult to cultivate, and 16S rRNA gene sequencing or similar methods have become of major importance to allow species identification and clinical diagnosis. Here, we report 2 patients with disease likely caused by a novel mycobacterial species that could not been previously cultivated. Diagnosis relied on molecular identification of acid-fast organisms in tissues. We also briefly review 2 similar cases published previously and note that all 4 known patients were from central Europe.

A 43-year-old woman without evidence of immunodeficiency reported recurrent episodes of dysuria, hematuria, and abdominal discomfort for >1 year. On cystoscopy, a hyperemic bladder with yellow plaques was observed. Biopsy of the plaques showed granulomatous infiltration of histiocytes. No definite diagnosis was made, and symptomatic relief occurred after a trial of empiric antimicrobial drug therapy. When the patient sought treatment again with persistent abdominal discomfort, endoscopy showed a lesion in the stomach that resembled a healed ulcer and numerous elevated yellow plaques throughout the colon and ileum. Microscopically, a granulomatous inflammation with macrophages filled with many acid-fast rods was seen, but mycobacterial growth did not occur in different media or in a guinea pig. Antituberculous treatment was initiated, and the patient slowly improved. A repeat colonoscopy showed fewer and smaller lesions. Efforts to culture the organism from biopsy specimens were again unsuccessful (different solid and liquid media, blood or chocolate agar, guinea pig, Balb/c mice). Sequencing of polymerase chain reaction (PCR) products of the 16S rRNA gene (*3*) from the organism represented a previously unknown mycobacterial species (EMBL DNA database: accession number Z50172). "*Mycobacterium tilburgii*" was proposed as the designation of this species because the novel mycobacterium was identified in the city of Tilburg (*4*). Retrospective analysis of the initial bladder biopsy specimen and of 2 lymph nodes taken during abdominal surgery (which became necessary because of a complicating ileal obstruction) confirmed the presence of "*M. tilburgii*" 16S rRNA gene sequences. All samples yielding PCR fragments hybridized with a "*M. tilburgii*"–specific biotinylated DNA probe.

A 34-year-old AIDS patient sought treatment for involuntary weight loss. Endoscopy showed white superficial embossments of the duodenal mucosa. Biopsy specimens were negative for acid-fast bacteria and mycobacterial growth (again, different solid and liquid media, extended incubation periods). Antiretroviral therapy was begun, but asthenia, persistent fever, diarrhea, vomiting, and cachexia developed. Repeat endoscopy showed yellow, plaquelike lesions in the duodenum (online Appendix Figure; available at http://www.cdc.gov/ncidod/EID/vol12no03/05-1139-G1.htm) and esophagus with periodic acid–Schiff (PAS) and acid-fast intracellular bacteria that were nonculturable. PCR of 16S rRNA gene (*3*) confirmed the presence of mycobacterial DNA; sequencing showed 100% homology to the

species with the proposed name "*M. tilburgii*" (*4*). Retrospectively, "*M. tilburgii*" 16S rRNA was present in the initial duodenal biopsy specimen, when no mycobacteria were detected microscopically. Combination therapy led to a gradual disappearance of fever and diarrhea. The patient's weight increased, and the lesions disappeared (Table). Control endoscopy results were unremarkable, although duodenal biopsy specimens showed PAS-positive material. Mycobacterial DNA was no longer detectable.

We believe that the identified acid-fast organism named "*M. tilburgii*" was the causative agent of illness in the 2 patients. First, the clinical and histopathologic appearance of the lesions is compatible with mycobacterial infection. Second, the finding that the macrophages in these lesions contained large numbers of acid-fast bacteria supports the conclusion that mycobacterial infection caused the lesion. Third, the involvement of normally sterile locations such as lymph nodes, and the improvement after antimycobacterial therapy with disappearance of the lesions, acid-fast organisms, and mycobacterial DNA supports a causative role of the organism rather than a bystander role.

Attempts to culture the organism from biopsy specimens and resection materials remained unsuccessful, although specific requirements for organisms like *M. genavense,* a mycobacterium that also is not cultivable on regular mycobacterial media (*5*), were met. Diagnosis therefore had to rely on sequencing of PCR products. Based on 16S rRNA homology, "*M. tilburgii*" shows close relationship to *M. sherrisii,* a *M. simiae*–related mycobacterium (*6*) that probably corresponds to *Mycobacterium* strain IWGMT 90143 (accession no. X88906) (*7*). "*M. tilburgii*" also has a high homology to *Mycobacterium* sp. Murphy, a mycobacterial species that is considered the cause of canine lepra and that also has not been cultivated so far (*8*) (accession no. AF144747).

These 2 patients double the number of patients reported previously (Table). Both previously reported *M. tilburgii* patients had AIDS, 1 of whom had a disease similar to that of the second patient in this report (*9*). Taken together, 3 patients had gastrointestinal involvement with yellow mucosal plaques (Appendix Figure), and 1 patient had pulmonary nodules (*10*). One of the 4 patients did not have a detectable immunodeficiency, yet had gastrointestinal and urinary bladder lesions. Although complications occurred, treatment was successful in all 4 cases with usual anti–*Mycobacterium avium* complex therapy. Interestingly, all 4 patients so far are of middle-European descent. This species may be geographically confined, similar to the occurrence of *M. malmoense* in which most clinical isolates come from northern Europe (*1*).

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Table. Characteristics of patients with confirmed "*Mycobacterium tilburgii*" disease*

Sex (age, y)	Immuno-suppression	CD4 count (cell/µL)	Disease manifestation	Treatment (duration, mo)	Diagnosed in	Reference
F (45)	No	Normal	Bladder, intestinal lymphnodes, stomach, ileum, colon	Pyrazinamide, isoniazid, rifampin (5); ethambutol, ciprofloxacin, clarithromycin, rifampin (11); surgical resection	The Netherlands	This study
M (34)	AIDS	37	Esophagus, duodenum	Ethambutol, clarithromycin, rifabutin (5)	Germany	This study
M (35)	AIDS	20	Duodenum, abdominal lymph nodes	Ethambutol, clarithromycin, rifabutin (12†)	Germany	(*9*)
M (41)	AIDS	17	Pulmonary nodules	Surgical resection; ethambutol, ciprofloxacin, clarithromycin (6); ethambutol, clarithromycin (12‡)	Germany	(*10*)

*F, female; M, male.
†6 weeks ineffective.
‡ Ongoing.

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Infection and Autoimmunity

Yehuda Shoenfeld and Noel R. Rose, editors

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As the editors imply in their introduction, the relationship of infection and autoimmunity is complex, compelling, and best viewed as a physiologic process and potential consequence of normal immune recognition and immunoregulation. The editors boldly state that reading the chapters in this book brings one to the conclusion that all autoimmune diseases are infectious, until proven otherwise (my paraphrase). Add environmental triggers to the mix, and most investigators would agree.

The book is divided into 3 broad sections: mechanisms of autoimmunity; specific infectious agents and their associated autoimmune diseases; and, conversely, specific autoimmune diseases and their associated infectious agents. The chapters in the mechanisms section focus on particular mechanisms, and with 1 exception, are scholarly and well done. However, this section lacks a review or balanced discussion of the various mechanisms of autoimmunity and proof of causation. Fortunately, the first article in the pathogen section by Denman and Rager-Zisman provides an excellent overview. As with any compendium (56 chapters by more than 100 authors), the quality varies, but all are written by investigators who have made substantial contributions to the field. The book is recommended for clinical investigators with some background in infectious disease or immunology as a starting point and ready resource for the current state of knowledge in the field.

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World Class Parasites: Vol. X, Schistosomiasis

W. Evan Secor and Daniel G. Colley, editors

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Pages: 235; Price: US $129.00

This compendium of reviews on the human parasitic infection, schistosomiasis, is a timely and much-needed resource for both research investigators and clinical personnel in many scientific and medical fields. Schistosomiasis is a major global health problem that affects hundreds of millions of people. It has been called one of the "great neglected diseases" because of the relative lack of interest by the pharmaceutical industry in developing diagnostics, drugs, and vaccines for it. The parasites that cause this disease, several related species of blood flukes, are also fascinating subjects for biologic research because of their complexity, relatively long lifespan, and remarkable host-parasite biology. To paraphrase Daniel Colley, one of the editors: "Analysis of this infection has contributed as much to our understanding of the human immune response and host parasite interactions as science has contributed to the control of the disease."

What sets this book apart is its readability for audiences from diverse backgrounds. This is in large part due to the thoughtfulness the editors put into choosing authors and topics, and perhaps most importantly, the relative brevity of each chapter. This latter point cannot be overemphasized since many compendiums overwhelm the reader with lengthy and dense material. This book is, therefore, especially valuable as a resource for students, postdoctoral fellows, and first-time investigators in this field.

One obvious conclusion that a reader new to the field will take away from this book is the diversity of research in this field. This reflects, as the editors note, a newfound intellectual energy born of the successful (albeit fledgling) applications of molecular genetic techniques to the study of the host-parasite relationship in schistosomiasis. At the same time, a shortcoming of the book is the relative lack of attention to the biochemistry of schistosomes, which is a key foundation for understanding how the parasite ticks and identifying new targets for chemotherapy. The work of investigators like John Dalton and Conor Caffrey on schistosome digestion of host proteins, for example, gets bare mention. This may be an inevitable consequence of the authors coming from a background of schistosome immunology, which is well represented in the book.

Another shortcoming is probably inescapable. As a field like schistosomiasis research enters a period of rapid and exciting advances, it is difficult for a book to keep up with the most recently published work. For example, many insights into the host-parasite relationship are becoming clear from proteomics analysis by groups like that of Alan Wilson at the

University of York. Perhaps this criticism is actually reflecting good news as the field is reenergized.

Colley correctly points out that the key to the future of research on this disease, and new approaches to its control, lies in recruiting a new generation of scientists and clinicians with an interest in the disease. I have been struck by the number of graduate students and MD/PhD students who have expressed a renewed interest in schistosome research over the past year. Like Colley, I believe this reflects the exciting technologic advances and novel approaches to understand the biology of this fascinating organism that are beginning to show success. If we are right that a new generation of young scientists are testing the waters of schistosome research, this excellent compendium could not come at a better time.

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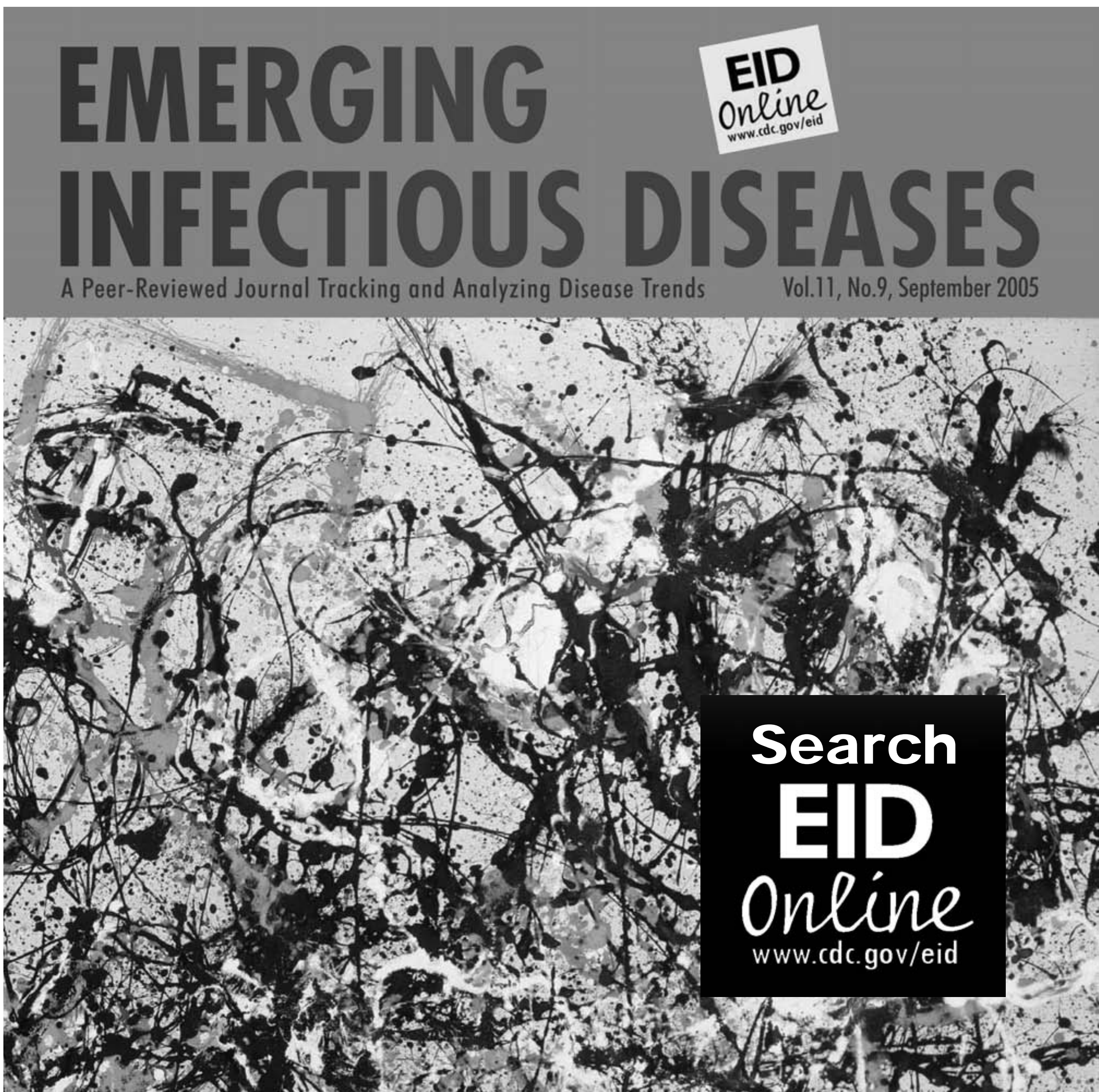

Rembrandt van Rijn (1606–1669). Scholar in His Study (1634) (detail).
Oil on canvas (141 cm × 135 cm). National Gallery in Prague, Czech Republic.

Different Strokes for Different Folks in Search of Truth

Polyxeni Potter*

For there will be hard data and they will be hard to understand
For the trivial will trap you and the important escape you
For the Committee will be unable to resolve the question
For there will be the arts
    and some will call them
    soft data
    whereas in fact they are the hard data
    by which our lives are lived

John Stone, "Gaudeamus Igitur: A Valediction"

The "ugly and plebeian face by which he was ill-favored, was accompanied by untidy and dirty clothes, since it was his custom, when working, to wipe his brushes on himself, and to do other things of a similar nature" (*1*), wrote Tuscan biographer Filippo Baldinucci about Rembrandt van Rijn. Though impatient with social conventions and lackadaisical with his own appearance, the artist became known for his impeccable rendition of character and sympathetic view of humanity in his portraits and historical paintings.

Rembrandt was born 400 years ago, during a period of unprecedented prosperity and peace in his native Holland. The son of a miller and a baker's daughter, he was, it was said, "of a different flour" (*2*). The eighth of nine children, he nonetheless received academic encouragement, attended Latin school, and entered the University of Leyden at age 14. Soon he left academic pursuits to become apprenticed to local painter Jacob van Swanenburgh, who worked in the tradition of Hieronymus Bosch (c. 1450–1516), and to study in Amsterdam under renowned history painter Pieter Lastman, who influenced his choice of biblical and mythologic subjects.

The greatest genius of what came to be called Holland's golden age, Rembrandt never traveled abroad. Instead, he absorbed influences indirectly, mostly from the followers of Caravaggio, the Italian master known for the originality of his realism and the use of light and dark (chiaroscuro) to convey intensity and drama (*3*). Rembrandt embraced the technique early, adapted it to his own boldly experimental application of paint and empathetic treatment of the world, and used it throughout his prolific career. Light and shadow became his vehicle for interpreting the human personality, establishing focus, creating mood and feeling, and transporting the viewer beyond the tangible world.

Exuberant self-confidence, native brusqueness, and general aversion to compromise, qualities that lent uniqueness and interest to his art, at times had a deleterious effect on Rembrandt's popularity with art patrons. "After it had become commonly known that whoever wanted to be portrayed by him had to sit to him for some two or three months," wrote Baldinucci, "there were few who came forward" (*4*). Once, while he painted the portrait of a couple with their children, he included in the work the corpse of his monkey, which had just died, to memorialize the pet.

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"The effect produced by the dead animal so impressed the artist that rather than remove it to satisfy his clients he left the work unfinished" (*4*). At a time when popular taste was turning to classical subjects, Rembrandt drew inspiration from "the humble, the rough, the decayed, the awkward and the heavy" (*5*), whom he painted not as they might have been but just as they were, providing ample clues to their temperament and predicament.

Though he achieved fame and fortune in his lifetime and had many students, among them Gerard Dou, Ferdinand Bol, and Carel Fabritius, the artist came to know personal tragedy. His days were marred with the deaths of his wife and children, disrupted by financial misfortune, and blighted by scandal. A series of self-portraits, more than 60 over 40 years, which trace his artistic and personal growth, show him conflicted, at times a beggar in rags, at times a prosperous man in opulent dress and gold chains. Wealth, respectability, recognition, and following failed to definitively establish his identity, either as esteemed member of society or rebellious outsider.

"I do not care so much for honor as I do for liberty" (*4*), Rembrandt asserted in defiance of his critics. Independence guided all aspects of his life. He was an eclectic art collector, a lover of exotic objects, an unpredictable operator. As his descriptions became less and less detailed and critics thought them sketchlike or unfinished, he retorted that a work is finished "when the master has achieved his intention in it" (*4*). In his later years, entirely oblivious to prevailing technique, he ignored those who thought his color "so heavily loaded that you could lift it from the floor by its nose" (*1*). Rather, his brushstrokes became thicker, his descriptions almost abstract in their reliance on light and dark to convey the "deepest and most lifelike emotion," trademark of his work.

Scholar in His Study, on this month's cover, pays tribute to the pursuit of knowledge, a subject visited often by Rembrandt (A Scholar, The Anatomy Lecture, Philosopher in Meditation, Aristotle Contemplating a Bust of Homer). The rich dark tones are characteristic of the artist's palette as is the interplay of light and shadow providing depth, contrast, and focus. The scholar, placed in the immediate foreground and viewed slightly from below, appears imposing and monumental (*6*). The thick folio lighted against rich brocade, the globe, the books, the surprised, even pained, response to what seems outside interruption imply that the study is of consequence and time of the essence.

Rembrandt's bias is reflected in the respectful rendering of the scholar's appearance. The coat of luxurious velvet embroidered with gold and trimmed with fur, the fantastic head gear, the fine hands suggest elegance, style, status. Despite the symbolism of aging (pallor framed by gray curls, furrowed brow, lusterless eyes inside dark circles, prominent nasal labial lines, fading features), the conjured portrait is of beauty, refinement, dignity, nobility, perseverance, intellectual cultivation. This elder scholar is no old fool.

In the secluded corner where Rembrandt has placed him, the scholar seeks what Aristotle believed all humans naturally desire, knowledge. "Clearly," he wrote in his Metaphysics, "…it is for no extrinsic advantage that we seek this knowledge…since it alone exists for itself" (*7*). In the old volumes stacked in front of him, the scholar searches for the truth about human existence, suffering, danger, hunger, disease, survival, knowing that life slips by before the task is done.

Rembrandt himself searched for the truth in the subjects he painted, in the common people whose complexity he sought to capture. And the penetrating analysis and contemplation characteristic of his self-portraits show no less than compulsion to know himself. His work expanded the world of knowledge, for he did not paint semblance alone. He saw, recognized, and expressed inner values and ideas, universal human traits, natural phenomena; explored, understood, and conveyed emotions; and defined, communicated, and commemorated all these. Piece by piece, in individual paintings and collectively in his life's work, he observed and recorded morsels of truth, seeking to understand and elucidate it.

Whereas his scholar dwelled on words, Rembrandt used color and brushstrokes. For these, along with numbers, notes on the staff, or sheer speculation are the tools for exploring the universe. And so it goes with science and public health. In isolation, like Rembrandt's scholar, in the laboratory, or in the field, public health workers search too, observing, recognizing and meticulously recording relevant information, surveying, delving into the unseen and implied, expanding knowledge.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the April issue for the following topics:

Potential Arbovirus Emergence Implications for the United Kingdom

Confronting Zoonoses, Linking Human and Veterinary Medicine

Coronavirus HKU1 Infection in the United States

Recently Acquired *Toxoplasma gondii* Infection, Brazil

Implications for Spread of Lyme Borreliosis

Reducing *Legionella* Colonization of Water Systems with Monochloramine

Emerging Pediatric HIV Epidemic Related to Migration

Encephalitic West Nile Virus in Central Europe

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

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

Prospects for a Universal Influenza Virus Vaccine

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

Identifying Influenza Viruses with Resequencing Microarrays

Animals as Sentinels of Bioterrorism

Complete list of articles in the April issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

March 16–18, 2006
International Conference on Women and Infectious Diseases: Progress in Science and Action
Marriott Marquis Hotel
Atlanta, GA, USA
http://www.womenshealthconf.org

March 19–22, 2006
International Conference on Emerging Infectious Diseases 2006
Marriott Marquis Hotel
Atlanta, GA, USA
http://www.iceid.org

March 22–24, 2006
International Symposium on Emerging Zoonoses
Medical and Veterinary Partnerships To Address Global Challenges
Marriott Marquis Hotel
Atlanta, GA, USA
http://www.isezconference.org

May 19–23, 2006
Council of Science Editors 49th Annual Meeting
Hyatt Regency Tampa
Tampa, FL, USA
http://www.councilscienceeditors.org

June 17–22, 2006
Negative Strand Viruses 2006: Thirteenth International Conference on Negative Strand Viruses
Salamanca, Spain
Contact: 404-728-0564 or meeting@nsv2006.org
http://www.nsv2006.org

June 25–29, 2006
ISHAM 2006 (International Society for Human and Animal Mycology)
Palais des Congrès
Paris, France
Contact: 770-751-7332 or c.chase@imedex.com
http://www.imedex.com/calendars/infectiousdisease.htm

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EMERGING INFECTIOUS DISEASES

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

* ★ New infections resulting from changes or evolution of existing organisms.
* ★ Known infections spreading to new geographic areas or populations.
* ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
* ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   * ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   * ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
   * ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   * ★ Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   * ★ Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   * ★ Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

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February 2006

EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.