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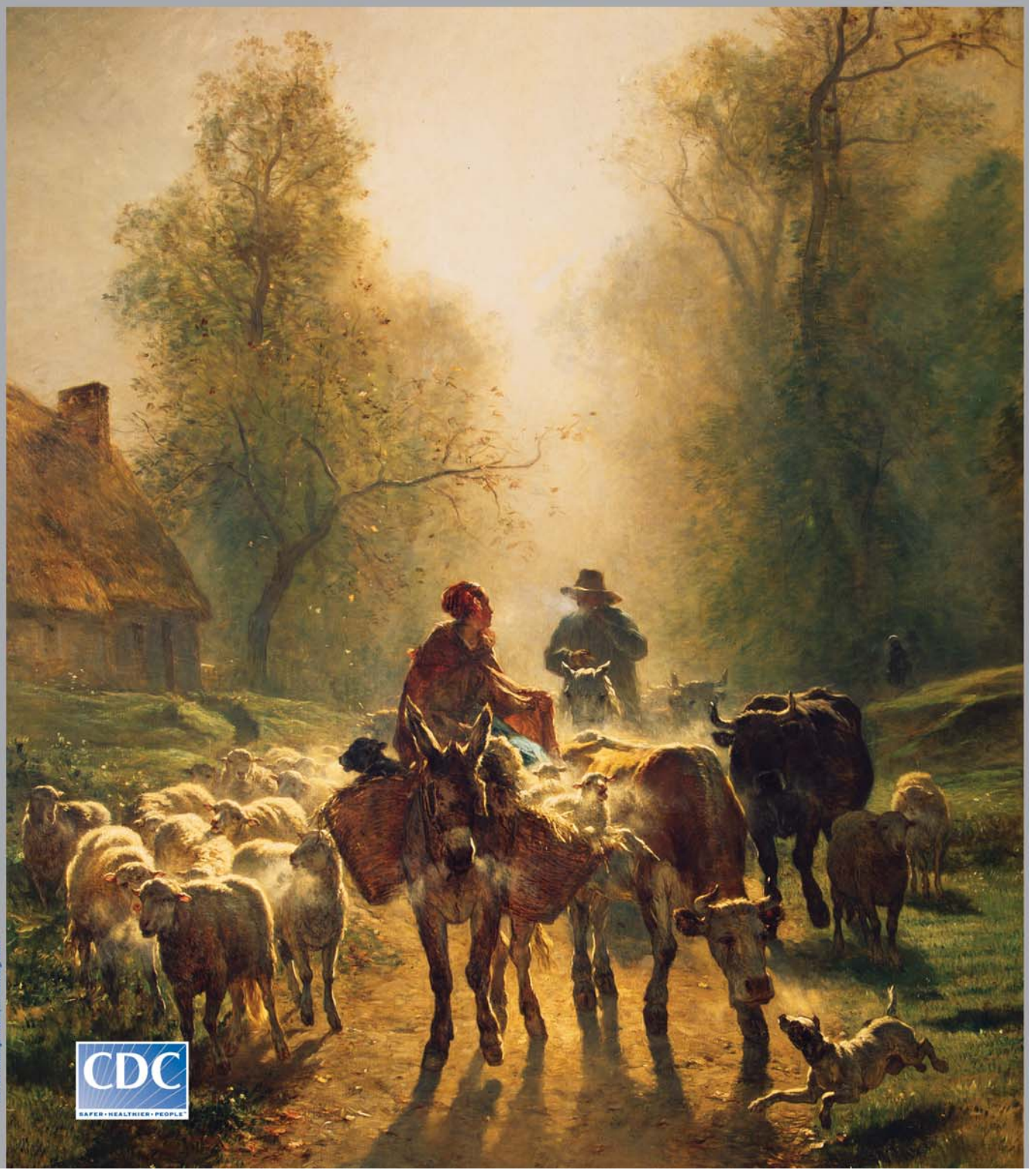

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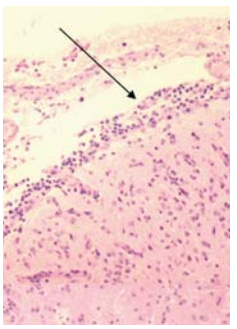
Research



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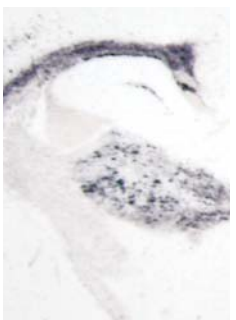

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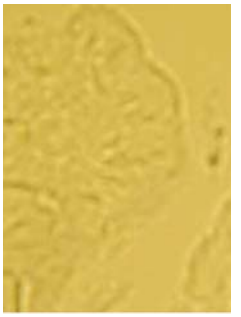

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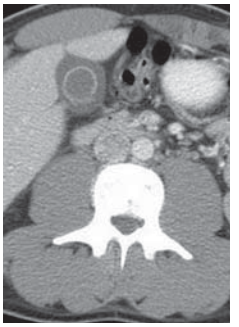

Letters

Book Review

News & Notes

Ecologic Immunology of Avian Influenza (H5N1) in Migratory Birds

Thomas P. Weber* and Nikolaos I. Stilianakis*†

The claim that migratory birds are responsible for the long-distance spread of highly pathogenic avian influenza viruses of subtype H5N1 rests on the assumption that infected wild birds can remain asymptomatic and migrate long distances unhampered. We critically assess this claim from the perspective of ecologic immunology, a research field that analyzes immune function in an ecologic, physiologic, and evolutionary context. Long-distance migration is one of the most demanding activities in the animal world. We show that several studies demonstrate that such prolonged, intense exercise leads to immunosuppression and that migratory performance is negatively affected by infections. These findings make it unlikely that wild birds can spread the virus along established long-distance migration pathways. However, infected, symptomatic wild birds may act as vectors over shorter distances, as appears to have occurred in Europe in early 2006.

Since its appearance in 1996 in a domestic goose in Guangdong Province, People's Republic of China, highly pathogenic avian influenza (HPAI) caused by a virus of subtype H5N1 has repeatedly been portrayed as the most prominent emerging disease threat faced by humanity. In addition to its high mortality rate for infected humans (currently 60%), a worrisome aspect of Asian lineage HPAI (H5N1) is its rapid spread from East Asia to Central Asia, Europe, and Africa in 2005–2006. In 2006–2007, Southeast Asia remained the geographic center of outbreaks in animals and humans. Migratory birds as well as trade involving live poultry and poultry products have been suggested as the most likely causes of dispersal of the virus (*1*–*3*). Several outbreaks in Central Asia and Europe of HPAI (H5N1) among wild bird populations that were apparently not in contact with domestic birds led to an increased interest in the potential role of wild migratory birds in the long-distance dispersal of the virus.

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Despite intensive research, the means by which this spread was accomplished have remained extraordinarily controversial. The divisiveness of this issue illustrates the point that an evaluation of emerging disease threats requires a broad interdisciplinary approach (*4*). It is thus disappointing that ornithologic knowledge and methods have not figured prominently in many high-profile studies that have shaped scientific, public, and political perceptions of the threat posed by HPAI (H5N1). Premature verdicts can have serious consequences. The view that disease transmission between wild birds and domestic poultry and humans is likely can seriously undermine conservation efforts concerning threatened migratory birds by eroding tolerance of what the public is led to believe are potential disease reservoirs.

We agree with Yasué et al. (*5*), who considered data on which migratory birds are considered responsible for long-distance spread of HPAI (H5N1) to be incomplete, inadequate, and often incorrect. For example, in a large number of cases involving wild birds in 2005 and early 2006, the Organisation Mondiale de la Santé Animale (Paris, France) did not report the species concerned. Lack of knowledge of the species involved in outbreaks among wild birds is just the tip of the iceberg. Even if species, age, and sex of affected birds were recorded correctly, many other interpretative issues often emerge. The ecology of infectious diseases and the immune system is an innovative field that has stimulated the attention and interest of ecologists (*6*) but is still struggling to be appreciated by the biomedical community. The field relies on fundamental information on the natural history and evolutionary ecology of the pathogens and hosts involved. Work on the natural history of avian migrants is published mainly in journals that easily escape the attention of veterinarians, virologists, epidemiologists, and molecular biologists. Relevant findings published in ecologic or physiologic journals are also easily missed by the scientists who deal most closely with avian influenza. An additional problem is that many important phenomena

in avian movements are not well researched, e.g., movements caused by cold weather and migratory connectivity.

Yasué et al. (*5*) and Feare and Yasué (*7*) have reported numerous problems with the soundness of many results concerning the involvement of wild birds in the spread of avian influenza. We complement these criticisms by concentrating on the neglected topic of seasonal (and shorter term) variation in the physiology of bird migration and consider how this variation might affect and be affected by immunocompetence. The immune function of migratory birds has so far received little attention in relation to avian influenza. We present pertinent and representative findings in this field. We argue that the considerable physiologic stresses of long-distance flights cast some doubts on the assumption that migratory birds are capable of spreading HPAI (H5N1) on a continental and transcontinental scale.

Ecologic Immunology of Migratory Birds

The hypothesis that migratory birds can transport HPAI (H5N1) over long distances rests on the assumption that some infected, virus-shedding wild birds show no or only mild symptoms and migrate long distances unhampered. There has been no direct test of this assumption, but several findings from ecologic immunology and exercise physiology studies are not compatible with this conjecture.

The immune system operates in a complex physiologic and ecologic context. The hormonal and nutritional states of an animal influence the functions of the immune system (*8*,*9*). These states are, in turn, affected by ecologic factors such as food supply, density of competitors and predators, energy expenditure, and injury. The fundamental idea of ecologic immunology is that maintaining a responsive immune system and mounting an immune response are energetically and nutritionally costly and that these costs have to be balanced against other expenses, such as reproduction, molting, growth, and development, that contribute to an animal's fitness (*6*,*10*). Thus, it is not only the direct negative effects of parasites that determine the consequences of an infection, but also the costs of the immune response. These costs are likely to become visible in situations in which animals are resource-limited. Animals might, for example, allocate more resources to immune function if challenged by an infection and expend less energy in other activities. Caring for young is energy-demanding, and activation of the immune response during breeding results in lower reproductive success or parental effort (*11*). Birds give up some of their current reproductive success to safeguard their survival and expected future reproductive success. Activating the immune system without being challenged by parasites can be costly. In a laboratory experiment with bumblebees (*Bombus terrestris*), Moret and Schmid-Hempel (*12*) showed that activation of the immune system of starved bumblebees resulted in lower survival rates. Hanssen et al. (*13*) reported similar results with eiders (*Somateria mollissima*, a migratory sea duck).

Long-distance migration is one of the most demanding physiologic activities in the animal world, and an adaptive resource allocation between concurrent physiologic processes likely occurs. Birds migrate for hours or even days at extremely high metabolic rates. During long flights, they can sustain up to 10× the basal metabolic rate. The bar-tailed godwit (*Limosa lapponica baueri*) may fly 6,000–8,600 km nonstop from New Zealand to stopover locations in Southeast Asia (*14*). Ducks generally travel shorter distances between stopover sites. However, because of their heavier bodies and shorter wings, ducks are less dynamically efficient and probably experience physiologic stress during their shorter migratory flights. The periods between flights are sometimes called resting phases, but this is clearly a misnomer. These are periods of frantic energy acquisition and physical recovery. During these stopovers, birds increase their body weight by 30%–50% of their lean mass in a few days with mainly fat to fuel the next step in their journey. Birds have evolved physiologic and behavioral adaptations to deal with these extreme demands of both energy expenditure and acquisition. Birds, especially those that migrate between widely separated stopover sites, adjust to these demands by regularly and repeatedly rebuilding their bodies. They increase the size of the digestive system and decrease flight muscle mass in refueling periods, and they go through the opposite adjustments before departure (*15*).

Migratory birds are well-adapted feeding and flying machines, but the exertion involved still takes its physiologic toll. Guglielmo et al. (*16*) reported that migratory flights result in muscle damage. Macrophages and other phagocytic cells invade the injured muscle cells and remove them. Migration and channeling of resources from the immune system can release latent infections in songbirds (*17*). Figuerola and Green (*18*) showed that the number of parasite species or genera reported per migratory waterfowl host species is positively related to migration distance. However, to infer that birds that migrate long distances are affected disproportionately by parasites, it would be necessary to show that they host more parasite species from each geographic region they pass through than resident waterfowl from the respective region.

Migratory birds have also evolved mechanisms to cope with a greater diversity of parasites than resident species. Møller and Erritzøe (*19*) found that migratory birds have larger immune defense organs than closely related nonmigratory birds. Owen and Moore (*20*) showed that 3 species of thrushes migrating through mainland America (only flying at night and resting and feeding during the day) are immunocompromised during spring and autumn migration. In humans, postexercise immune function depression is most pronounced when exercise is continuous, prolonged,

of moderate-to-high intensity, and performed without food intake (*21*). However, whether similar mechanisms linking exercise and immune function also apply to birds is not known.

These representative studies demonstrate that physiologic demands of long-distance migration can suppress the immune system. Far less information is available, however, on 1 important aspect: how do infected birds perform during long-distance migration? Møller et al. (*22*) showed that barn swallows (*Hirundo rustica*) with large energy reserves maintain better immune function during migration, clear ectoparasites and blood parasites more effectively, and arrive earlier at breeding grounds (which is an important determinant of reproductive success) than birds with poor energy reserves. Some indirect evidence shows how exercise during migration, infection, and immune responses could interact. As mentioned, Hanssen et al. (*13*) demonstrated that in eiders, immune system activation can have severe negative consequences. These researchers injected females with 3 different nonpathogenic antigens (sheep erythrocytes, diphtheria toxoid, and tetanus toxoid) early in their incubation period. Mounting of a humoral immune response against these antigens decreased the return rate to the breeding grounds in northern Norway from 72% to 27%, which implied a high cost of the immune response. However, it is not clear from these results whether birds died during migration or during overwintering or whether the reduced return rate reflected only failure of birds to migrate back to their breeding grounds. Also, the demands of thermoregulation can be substantial. Liu et al. (*23*) reported correlations between sudden temperature decreases and activation of latent infection with influenza A virus.

The most direct evidence of interaction between demands of migratory flights and infections was reported by van Gils et al. (*24*). These authors found that Bewick's swans (*Cygnus columbianus bewickii*) infected with low pathogenic avian influenza A viruses of the subtypes H6N2 and H6N8 performed more poorly in terms of foraging and migratory behavior than uninfected birds (including birds that had recovered from a previous infection). Infected birds had lower bite rates, took more time to deposit the energy reserves required for migration, departed later, and made shorter journeys. The researchers suspect that the swans might have traded off energy invested in immune defense against energy invested in rebuilding their bodies for efficient fuel deposition and flight. However, as van Gils et al. (*24*) also reported, only a controlled experimental study can establish whether this hypothesis is plausible. However, such a study will probably never be done because release of the H5N1 subtype of HPAI virus into the wild is banned. A large number of studies of domestic and laboratory mammals show that many bacterial, viral, and parasitic infections lead to anorexia in the host (*25*). The findings reported by van Gils et al. (*24*) are consistent with known patterns of infection-induced anorexia in mammals.

These findings do not offer a definite rebuttal, but they cast some serious doubts on the frequently repeated claim that wild birds can easily act as long-distance vectors for influenza A viruses. However, some caveats need to be addressed that make any quick judgment impossible. The study by van Gils et al. (*24*) had a low sample size of infected birds. Furthermore, it was conducted during spring migration. In many migratory species, spring and autumn migration are likely to occur under different conditions. The considerable stress of spring migration may be amplified by energetically costly flights undertaken when food resources are often still scarce along the migratory route, as well as at breeding grounds at the time of arrival (*26*,*27*). After arrival at breeding grounds, the birds' energy must be invested in display and, in females, in egg production. In autumn, feeding conditions are generally better along migratory routes. If autumn migration, when infections with influenza A viruses are more prevalent in waterfowl, proceeds under more benign feeding conditions, the immune system of birds might be able to clear infections more effectively. This may mean that the birds can clear infections quickly or that the infection is controlled by the immune system but not entirely cleared, and virus-shedding still occurs. Hasselquist et al. (*28*) showed in a wind-tunnel experiment with the red knot (*Calidris canutus*), a long-distance migratory bird, that long flights did not influence immune responses. However, they also found that some birds with low antibody responses against tetanus refused to fly. This suggests that there is a trade-off between the demands of different physiologic systems and that only birds in good condition with energy to spare may be willing to expend this energy.

Sparse findings on immunocompetence and exercise in migratory birds do not decisively rule out the possibility that HPAI (H5N1) may be transported relatively short distances by wild birds. That wintering birds are leaving areas with cold weather does not necessarily imply stressful long flights and the physiologic adjustments that accompany long-distance migration. Even birds incapacitated by an infection may therefore manage to escape harsh weather. However, causes and consequences of cold weather movements have not been investigated in sufficient detail (*29*). An analysis by Feare (*30*) supported the view that long-distance spread of virus by migratory birds is unlikely but short-distance spread is possible. Feare (*30*) examined all known major outbreaks in wild birds and concluded that most occurrences reflect local acquisition from a contaminated source, followed by rapid death nearby. Outbreaks in Europe in 2006 indicate that infected wild birds can travel a limited distance before dying of influenza and can pass the virus to other wild or domestic birds.

Conclusion

No convincing evidence has yet shown that infected, asymptomatic wild birds can or do carry influenza virus along established, seasonal long-distance migration routes. Even infected dying swans do not shed HPAI (H5N1) in large quantities; swans may thus constitute an end host and not be carriers or efficient transmitters (*31*,*32*). The controversies surrounding HPAI (H5N1) and its likely mode of spread show how little is known about some important topics in the field of emerging infectious diseases. These topics include epidemiology of parasites with highly mobile host species and function of the immune system of these highly mobile host species who experience diverse climatic and ecologic conditions and variable parasite faunas during their annual cycle.

Recent work on the role of migratory Saiga antelopes in livestock disease epidemiology has shown how host movement, multiple host species, and temporal and climatic variation must be included in population dynamics models of parasites (*33*). However, studies must go beyond such necessary and welcome modeling efforts. Research in ecologic immunology has shown that the functionality of the immune system has to be considered in an ecologic and evolutionary life-history context. The immune system shows complex and, from an evolutionary point of view, often adaptive dynamics with multifaceted interactions with nutritional, hormonal, and energetic states and other physiologic processes. However, ecologic immunology is a discipline in its infancy and still often works with rather simplistic ideas. For example, the immune system is often implicitly assumed to be a unified system that competes with other physiologic processes for energy and nutrients. Long and Nanthakumar (*34*) showed this to be an unrealistic and naive assumption; they emphasize the necessity of considering the differential effects of energy or nutrient stress on specific subcomponents of the immune system.

It therefore remains a critical task to research the capacities and limitations of the immune system in wild birds under natural conditions. Only then will it be possible to judge how results from laboratory experiments can be transferred to natural situations. For example, Hulse-Post et al. (*35*) have shown that HPAI (H5N1) evolves to lowered pathogenicity in captive laboratory-maintained mallards (*Anas platyrhynchos*) but remains highly lethal for chickens. This finding suggests that ducks may act as asymptomatic carriers. However, it remains unclear whether free-living, migratory wild ducks facing stressors such as food shortages or long flights are as immunocompetent as their laboratory counterparts or whether virus evolution takes the same course under such conditions. The commercial movement of asymptomatically infected domestic ducks, often for pest control reasons and over longs distances, could be a mechanism of spread.

Two of the major challenges in the 21st century are emerging diseases and the protection of biodiversity. Sustainable solutions for these challenges can be fostered only in a respectful interdisciplinary atmosphere. Migratory birds are already affected by habitat destruction and climate change; alarmist statements blaming migrants for the spread of an emerging disease with pandemic potential and ignoring or underplaying the role of the poultry industry do not do justice to the complexity of the issues involved (*36*,*37*).

Dr Weber works for the European Commission's Joint Research Centre (JRC) in Ispra, Italy. His research interests include modeling of infectious diseases and evolutionary ecology and physiology of avian migration

Dr Stilianakis is a biomathematician who works at the European Commission JRC and an assistant professor of epidemiology and biomathematics at the University of Erlangen-Nürnberg Medical School in Erlangen, Germany. His research interests include development of models for pathogenesis and epidemiology of infectious diseases.

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Risk Factors for Colonization with Extended-Spectrum β-Lactamase–Producing Bacteria and Intensive Care Unit Admission

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Extended-spectrum β-lactamase (ESBL)–producing bacteria are emerging pathogens. To analyze risk factors for colonization with ESBL-producing bacteria at intensive care unit (ICU) admission, we conducted a prospective study of a 3.5-year cohort of patients admitted to medical and surgical ICUs at the University of Maryland Medical Center. Over the study period, admission cultures were obtained from 5,209 patients. Of these, 117 were colonized with ESBL-producing *Escherichia coli* and *Klebsiella* spp., and 29 (25%) had a subsequent ESBL-positive clinical culture. Multivariable analysis showed the following to be statistically associated with ESBL colonization at admission: piperacillin-tazobactam (odds ratio [OR] 2.05, 95% confidence interval [CI] 1.36–3.10), vancomycin (OR 2.11, 95% CI 1.34–3.31), age >60 years (OR 1.79, 95% CI 1.24–2.60), and chronic disease score (OR 1.15; 95% CI 1.04–1.27). Coexisting conditions and previous antimicrobial drug exposure are thus predictive of colonization, and a large percentage of these patients have subsequent positive clinical cultures for ESBL-producing bacteria.

Extended-spectrum β-lactamase (ESBL)–producing gram-negative bacteria are emerging pathogens. Clinicians, microbiologists, infection control practitioners, and hospital epidemiologists are concerned about ESBL-producing bacteria because of the increasing incidence of such infections, the limitations of effective antimicrobial drug therapy, and adverse patient outcomes (*1–5*).

Research conducted to date has focused on identifying risk factors for colonization with multidrug-resistant, gram-positive bacteria. In contrast, little research has been conducted to identify the risk factors for colonization with gram-negative multidrug-resistant bacteria in nonoutbreak settings. To our knowledge, no study of the magnitude of our study has been conducted, nor have any studies based in the United States sought to identify risk factors for colonization with ESBL-producing bacteria on admission to an intensive care unit (ICU).

The primary objective of our study was to identify factors predictive of colonization with ESBL-producing bacteria at admission to an intensive care unit (ICU). In addition, we identified the percentage of patients colonized with ESBL-producing bacteria who had a subsequent positive clinical culture for the same species of ESBL-producing bacteria. Understanding risk factors for colonization is important for several reasons. First, understanding the potential causal mechanisms of colonization can lead to successful infection control, involving antimicrobial stewardship and public health interventions aimed at controlling the emergence of ESBL-producing bacteria. Second, such knowledge can help identify which patients should be receiving empiric ESBL-targeted antimicrobial therapy. Some hospitals have used active surveillance culturing for antimicrobial drug–resistant, gram-negative bacteria to help guide empiric therapy (*6*).

Materials and Methods

Study Population and Sample Collection

We conducted a prospective cohort study of patients admitted to either the surgical or medical ICU at the University of Maryland Medical Center from September 1,

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2001, through June 1, 2005. Descriptions of the hospital and the ICUs are reported in other publications (*7*,*8*). During the study period, on average, 8.6 clinical cultures per month were positive for ESBL-producing bacteria. No outbreaks of ESBL-producing bacteria were found among clinical cultures based on control process charting. No additional infection control precautions were used for patients with ESBL-producing bacteria on clinical culture. ESBL surveillance culture results were not given to physicians or nurses. Contact isolation precautions were applied for patients with vancomycin-resistant enterococci or methicillin-resistant *Staphylococcus aureus* infections.

During the study period, nurses obtained perianal specimens for culture from all ICU patients within 48 hours of ICU admission. All patients who had admission culture results were included in this study. Patients with multiple admissions to either of the ICUs during the study period were allowed to enter the cohort of at-risk patients multiple times, as long as they were not positive for ESBL-producing bacteria on any prior admissions (because patients remain at risk for ESBL-producing bacteria on each subsequent admission). This study was approved by the Institutional Review Board of the University of Maryland, Baltimore. Informed consent was not required by the Institutional Review Board because perianal specimens were cultured as part of infection control quality improvement involving active surveillance culturing for vancomycin-resistant enterococci.

Microbiologic Methods

The perianal cultures were processed for ESBL-producing bacteria in real time as the specimens were collected. The perianal cultures were first screened for potential ESBL-producing bacteria by plating onto MacConkey agar (Remel, Lenexa, KS, USA) with 2 μg/mL of ceftazidime added to the cooled agar before the plates were poured (*9*). Plates were incubated at 37°C for 24 to 48 hours. Lactose-fermenting colonies growing on the ceftazidime-containing plates were identified as *Escherichia coli* or *Klebsiella* species by using API 20E identification strips (bioMérieux Vitek, Inc., Hazelwood, MO, USA). All *E. coli* and *Klebsiella* isolates underwent ESBL confirmatory testing by disk diffusion for ceftazidime and cefotaxime with and without clavulanic acid as recommended by the Clinical Laboratory Standards Institute's guidelines (*10*).

Data Collection and Variables

For all patients included in the study, we collected data regarding the patient's previous hospital antimicrobial drug exposures, length of hospitalization before ICU admission, coexisting conditions, previous positive cultures, and other hospitalization-related and demographic information. Antimicrobial drug exposures were assessed in the period between hospital admission and ICU admission. Antimicrobial drugs were analyzed as binary variables; if an antimicrobial drug was received during the period defined above, it was classified as having been received independent of the number of doses received. Duration of antimicrobial drug exposure was not analyzed. Coexisting conditions were assessed by the Charlson Comorbidity Index, the Chronic Disease Score (CDS), and the infectious disease–specific CDS (CDS-ID) (*11*–*13*).

Initial bivariable statistical comparisons were conducted by using the χ^2 test for categorical data and the Student *t* test or Wilcoxon test for continuous data. Continuous variables that were not normally distributed were categorized for the purpose of multivariable analyses. To identify patient characteristics associated with colonization by an ESBL-producing bacterium on ICU admission, we used multivariable logistic regression. Because patients were allowed to enter into the study multiple times, we also assessed the need to control for the correlated error structure of the data. All variables that were associated with ESBL colonization in the bivariable analysis at the $p<0.1$ level were included in the model-building stages of the multivariable analysis. A stepwise model building method was used. Variables were retained in the final model if they were significant at a $p<0.05$ level or if they were observed to have a confounding effect on the association between another predictor and ESBL colonization status. A confounding effect was defined as a change in the model coefficient by >10%. An additional bivariable statistical analysis was performed to identify risk factors for subsequent clinical culture positivity with the same species of ESBL-producing bacteria among the cohort of patients colonized with an ESBL-producing bacteria. We calculated the C statistic of the final model. The C statistic reports values from 0.5 (indicating no predictive power) to 1.0 (indicating perfect prediction). In addition, we calculated the sensitivity, specificity, positive predictive value, and negative predictive value for patients with or without all dichotomous variables in the final model. Statistical analysis was performed with SAS Version 9.1 (SAS Institute, Cary, NC, USA).

Results

During the study period, 5,209 (84%) admitted patients had results of admission perianal cultures and were included in this study, 4,398 patients had 1 ICU admission, and 618 patients had repeat admissions. Ninety-one percent of the surveillance cultures were obtained within the first 12 hours of ICU admission. The cross-sectional patient cohort consisted of 2,096 (40%) admissions to the medical ICU and 3,113 (60%) admissions to the surgical ICU. The mean age of the patients was 55 years. The mean comorbidity score as measured by the CDS–ID was 2.73 and 2.42 as measured by the Charlson Comorbidity Index. Based

upon International Classification of Diseases, 9th revision (ICD-9) codes, 1,285 (25%) had diabetes, 1,344 (26%) had cancer, and 193 (4%) were HIV positive; 1,594 (31%) of patients had been transferred from another healthcare facility, and 1,693 (33%) had been previously admitted to the same hospital within the past year.

We examined patient characteristics, coexisting conditions, and previous antimicrobial drug exposures to identify factors potentially associated with colonization by an ESBL-producing bacterium on ICU admission (Table 1, bivariable analysis). Of 5,209 patient admissions, 117 (2%) patients were colonized by an ESBL-producing *E. coli* or *Klebsiella* species bacterium on ICU admission. Specifically, 76 (65%) patients were colonized by an ESBL-producing *E. coli,* 55 (47%) were colonized by an ESBL-producing *Klebsiella* species, and 14 (12%) patients were colonized by both. Stratified bivariable analyses by organism are as follows: for *E. coli*, zosyn (odds ratio [OR] 1.93; 95% confidence interval [CI] 1.17–3.18), vancomycin (OR 1.66, 95% CI, 0.91–3.03), age (OR 2.51, 95% CI 1.57–4.00), and coexisting conditions (OR 1.19, 95% CI 1.05–1.35); for *Klebsiella* spp., zosyn (OR 2.30, 95% CI 1.31–4.06), vancomycin (OR 3.91, 95% CI 2.21–6.91), age (OR 1.29, 95% CI 0.76–2.20), and coexisting conditions, as measured by CDS-ID (OR 1.12, 95% CI 0.96–1.31). Stratified analysis results for those patients who were in the hospital at least 24 hours before ICU admission are as follows: zosyn (OR 2.34, 95% CI, 1.11–4.94), vancomycin (OR 3.25, 95% CI 1.57–6.75), age (OR 1.39, 95% CI 0.68–2.86), and coexisting conditions as measured by CDS-ID (OR 95% CI 1.01–1.44).

The results of the final multivariable logistic regression analysis are shown in Table 2. Age >60 years (OR 1.79, 95% CI 1.24–2.60), coexisting conditions as measured by the CDS-ID (OR 1.15, 95% CI 1.04–1.27), in-hospital use of piperacillin-tazobactam (OR 2.05, 95% CI 1.36–3.10), and in-hospital use of vancomycin (OR 2.11, 95% CI 1.34–3.31) were all found to be independently associated with colonization by an ESBL-producing bacterium on admission to an ICU. No other antimicrobial drug was found to have a significant ($p<0.05$) effect in the final multivariable model. Note that we did not adjust for the correlated error structure of the data in the final analysis; because the correlation was low, this adjustment had little effect on our estimates (data not shown). The C statistic of the final model was 0.69. Patients categorized on the basis of the presence of all of the following dichotomous predictors of the final model (zosyn, vancomycin and age >60) yielded a sensitivity of 9.4%, specificity of 97.3%, positive predictive value of 7.3%, and negative predictive value of 97.9%.

For the 117 patients identified as colonized with ESBL-producing bacteria, we assessed their history of culture positivity with ESBL-producing bacteria as well as other antimicrobial drug–resistant bacteria (Table 3). Of the ESBL-colonized patients, 6 (5%) had positive clinical cultures for ESBL-producing bacteria during the same hospital admission but before ICU admission, and 29 (25%) had a subsequent ESBL-positive clinical culture from the time an ICU admission surveillance specimen was obtained for culture to the date of hospital discharge. The only risk factor that predicted subsequent positive ESBL clinical culture was the amount of time in the hospital between positive surveillance culture and hospital discharge (OR 1.03 per additional day, 95% CI 1.01–1.06). These 29 patients had 56 clinical cultures with ESBL-producing bacteria. The sources of the 56 clinical cultures positive for ESBL-producing bacteria were the following: 9 blood cultures, 17 sputum or bronchoscopy specimens, 10 urine cultures, 12 wound cultures, and 8 miscellaneous sources. Of 117 ESBL-colonized patients, 41 (35%) were known to have

Table 1. Potential predictors of colonization by an ESBL-producing bacterium on ICU admission*

Potential predictor	No. ESBL colonized (n = 117)	No. not ESBL colonized (n = 5,092)	p value†
Age, y (median, IQR)	62 (49–71)	56 (45–67)	<0.01
CDS (median, IQR)	8 (5–10)	8 (5–10)	0.20
CDS-ID (median, IQR)	3.21 (1.83–4.78)	2.83 (1.83–3.40)	<0.01
Sex, female, no. (%)	59 (50)	2,311 (45)	0.30
Antimicrobial drug exposures, no. (%)‡			
Quinolone	18 (15)	617 (12)	0.32
1st-generation cephalosporin	9 (8)	559 (11)	0.30
3rd-generation cephalosporin	7 (6)	293 (6)	0.84
Vancomycin	34 (29)	616 (12)	<0.01
Aminoglycoside	11 (9)	366 (7)	0.36
Piperacillin-tazobactam	50 (43)	1,090 (21)	<0.01
Cefepime	9 (8)	161 (3)	0.01
Imipenem	11 (9)	224 (4)	0.02

*ESBL, extended-spectrum β-lactamase; ICU, intensive care unit; IQR, interquartile range; CDS, Chronic Disease Score; CDS-ID, infectious disease–specific CDS.
†Fisher exact test for dichotomous predictors and Wilcoxon test for continuous predictors.
‡Antimicrobial drug exposures that occurred during the index hospital admission but before ICU admission.

Table 2. Independent predictors of ESBL-producing bacteria colonization in multivariable logistic regression model*

Predictor	OR	95% CI
Age >60	1.79	1.24, 2.60
CDS-ID	1.15	1.04, 1.27
Vancomycin†	2.11	1.34, 3.31
Piperacillin-tazobactam†	2.05	1.36, 3.10

*ESBL, extended-spectrum β-lactamase; OR, odds ratio; CI, confidence interval; CDS-ID, infectious disease–specific Chronic Disease Score.
†Antimicrobial drug exposures were assessed during the period between hospital admission and intensive care unit admission.

been previously infected or colonized with either methicillin-resistant *S. aureus* (MRSA) or vancomycin-resistant enterococci (VRE). Among the 5,092 patients not colonized with ESBL-producing bacteria, 33 (0.6%) had a subsequent positive ESBL clinical culture with the same bacterial species between the time of ICU admission surveillance culture to the date of hospital discharge.

Discussion

In this study, we identified risk factors for colonization with ESBL-producing *E. coli* and *Klebsiella* spp. at ICU admission. We identified age >60 years, comorbidity as measured by the CDS-ID, previous in-hospital piperacillin-tazobactam use (current admission), and previous present admission in-hospital vancomycin use (current admission) as independent risk factors. We also quantified the ESBL colonization/clinical culture positivity rate among these patients and addressed the question of whether patients colonized with ESBL had a history of colonization with MRSA and VRE.

The risk factors identified are potentially important because they can help determine which patients may need empiric antimicrobial drug therapy targeted to the ESBL-producing bacteria. Carbapenem antimicrobial agents may be preferred as empiric choice for patients at risk for ESBL-producing bacteria (*2*). We are not recommending that all patients with these risk factors receive empiric antimicrobial drug therapy targeted to ESBL-producing bacteria. However, among particular patients with the identified risk factors and levels of severity of infection that require empiric therapy, a choice of empiric therapy that includes coverage of ESBL-producing bacteria may be warranted. Thus, we recommend that for patients in ICUs with similar characteristics to the units in this study, physicians consider using antimicrobial agents targeted against ESBL-producing bacteria. These ESBL-targeted drugs should be considered when the physician chooses to prescribe an antimicrobial drug for situations such as fever of unknown origin, suspected pneumonia, or suspected bacteremia. In addition, these risk factors identified may be of use to hospital antimicrobial drug stewardship programs and pharmacy and therapeutics committees.

We hope that our risk factor study and other risk factor studies in the area of antimicrobial drug resistance will be used in future antimicrobial agent stewardship intervention studies and future infection control intervention studies. Previous risk factor studies have led to antimicrobial agent stewardship intervention studies aimed at controlling ESBL-producing bacteria (*14*,*15*). In the areas of pneumonia and neutropenia patients with fever, risk factors studies have successfully led to intervention studies that have affected national guidelines (*16*–*18*). Well-designed intervention studies, based on risk factor studies of antimicrobial drug resistance, can lead to more appropriate antimicrobial drug use, which will improve patient outcomes and decrease the emergence of antimicrobial drug resistance (*19*,*20*).

The risk factors identified may be causally related to the outcome of ESBL-colonization or may only be statistically associated. Age >60 years and the presence of coexisting conditions have validity and biologic plausibility for a causal association with colonization status (*1*,*9*,*21*). The identification of piperacillin-tazobactam and vancomycin as risk factors is more intriguing. Vancomycin and piperacillin-tazobactam are widely used at our tertiary-care hospital, the University of Maryland Medical Center, and thus may just be markers of ICU patients who require broad-spectrum antimicrobial coverage. However, understanding intestinal ecology and antimicrobial drug resistance is still in nascent stages. Vancomycin and piperacillin-tazobactam may be true causal risk factors for colonization with ESBL-producing bacteria. Piperacillin-tazobactam is believed to be effective against ESBL-producing bacteria only when the inoculum is low (*22*). Thus, with regard to the intestinal flora, piperacillin-tazobactam may not be effective at eradicating ESBL-producing bacteria due to inoculum effects and low intestinal concentration of piperacillin-tazobactam. Additionally, we were surprised by the identification

Table 3. History of culture positivity with antimicrobial drug–resistant bacteria among 117 patients colonized with ESBL-producing bacteria at ICU admission*

Drug-resistant bacteria	No. ESBL colonized (%)
ESBL-positive clinical cultures before ICU admission†	6 (5)
ESBL-positive clinical cultures after colonization	29 (25)
Methicillin-resistant *Staphylococcus aureus*†	25 (21)
Vancomycin-resistant enterococci‡	27 (23)

*ESBL, extended-spectrum β-lactamase; ICU, intensive care unit.
†Positive clinical cultures during the same hospital admission but before ICU admission.
‡Clinical or surveillance cultures at any time before ICU admission.

of piperacillin-tazobactam as a risk factor as some hospitals have adopted antimicrobial drug stewardship policies that have limited the prescribing of cephalosporins and increased the use of antimicrobial drugs, including piperacillin-tazobactam, in an effort to control ESBL-producing bacteria (*15*,*23*). Vancomycin may be a risk factor through relative decolonization of the normal flora through vancomycin exposure and then subsequent colonization with ESBL strains through horizontal transmission before ICU admission (*24*,*25*).

We found a ratio of colonization to clinical culture positivity that was the same order of magnitude as for VRE and MRSA (*26*–*29*). In addition, only 35% of patients with ESBL-colonization were previously known to be VRE or MRSA positive. These numbers and the local prevalence rate of ESBL-producing bacteria are important parameters in assessing the cost-effectiveness of active surveillance for ESBL-producing bacteria. Further work, including cost-effectiveness studies, needs to address whether active surveillance is beneficial for ESBL-producing bacteria.

Several studies. performed worldwide, have analyzed risk factors for colonization with multidrug resistant *Enterobacteriaceae*. Many studies have not analyzed the specific antimicrobial drug resistance mechanism and thus are not directly comparable to our study. A study from Canada determined that several antimicrobial drugs were risk factors for multidrug resistant *Enterobacteriaceae* (*30*). In contrast to our study, most of their isolates had AmpC as a resistance mechanism, and thus their study did not determine risk factors for ESBL-producing bacteria. A 4-year cohort study done in France determined the ESBL-producing bacteria colonization rate in 2 ICUs to be 0.97% and thus concluded that, in their setting, active surveillance was unlikely to be cost-effective (*31*). A study in Israel identified 26 (10.8%) of 241 patients tested by active surveillance as colonized with ESBL-producing bacteria. Risk factors identified in multivariable analysis were poor functional status, current antimicrobial drug use, chronic renal insufficiency, liver disease, and the use of histamine$_2$ receptor antagonists (*32*).

A limitation of our study is that we did not have access to records of the antimicrobial drugs that patients may have received as outpatients before their hospital admission. However, relevant to the question of empiric therapy, most intensive care clinicians do not have access to records of outpatient antimicrobial drug use when they are empirically choosing antimicrobial agents. Another limitation of the study is that we did not have access to the subsequent ESBL-positive clinical isolates and thus were unable to compare them by molecular epidemiologic methods, such as pulsed-field gel electrophoresis, to see whether they were identical to the ESBL-colonizing isolates identified previously. We did not perform chart review; thus, the subsequent clinical cultures with ESBL-producing bacteria could have represented either clinical infection or colonization, based on definitions from the Centers for Disease Control and Prevention (*33*). The use of ceftazidime in the screening agar may have caused the CTX-M family of β-lactamases to be missed. However, no CTX-M enzymes were detected in a sample of clinical isolates from the University of Maryland Medical School and the adjacent Veterans Affairs Medical Center from 2001 to 2002 (*34*).

In this study, we identified risk factors for ESBL-producing bacterial colonization among ICU patients. These data may be useful for identifying which patients may warrant empiric ESBL-targeted antimicrobial drug therapy. We also demonstrate that subsequent infections with ESBL-producing bacteria develop in a large percentage of ICU patients colonized with ESBL-producing bacteria.

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Occupational Risks during a Monkeypox Outbreak, Wisconsin, 2003

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We determined factors associated with occupational transmission in Wisconsin during the 2003 outbreak of prairie dog–associated monkeypox virus infections. Our investigation included active contact surveillance, exposure-related interviews, and a veterinary facility cohort study. We identified 19 confirmed, 5 probable, and 3 suspected cases. Rash, headache, sweats, and fever were reported by >80% of patients. Occupationally transmitted infections occurred in 12 veterinary staff, 2 pet store employees, and 2 animal distributors. The following were associated with illness: working directly with animal care (p = 0.002), being involved in prairie dog examination, caring for an animal within 6 feet of an ill prairie dog (p = 0.03), feeding an ill prairie dog (p = 0.002), and using an antihistamine (p = 0.04). Having never handled an ill prairie dog (p = 0.004) was protective. Veterinary staff used personal protective equipment sporadically. Our findings underscore the importance of standard veterinary infection-control guidelines.

During May–June 2003, an outbreak of monkeypox virus (MPXV) infections, initially detected in Wisconsin, occurred in the midwestern United States (*1*,*2*). These MPXV infections were the first to be reported outside of Africa and involved a West African viral strain (*1*,*3*). African rodents imported from Ghana were implicated in virus introduction in the United States (*2*,*4*–*7*). The African rodents had been transported and housed with native prairie dogs that were subsequently distributed as household pets in Wisconsin (*1*). Veterinary and pet store staff are at risk for potentially serious occupationally related infections (*8*–*18*). Early links between MPXV infections and prairie dog exposures in veterinary facilities and pet stores (*1*) led us to investigate occupationally related exposures.

We conducted an outbreak investigation and a veterinary staff cohort study to quantify and characterize all cases that occurred during the 2003 Wisconsin MPXV outbreak, identify protective and risk factors for occupationally transmitted infections, and determine veterinary work practices amenable to infection-control guidelines. Because both investigations were urgent outbreak control measures, no institutional review board approval or written consent was required or obtained.

Methods

Outbreak Investigation

The Wisconsin outbreak case definition (online Appendix, available from www.cdc.gov/EID/content/13/8/1150-app.htm) was similar to case definitions established by the Centers for Disease Control and Prevention (CDC) for human MPXV infection (*19*). Cases were classified as confirmed, probable, or suspected according to clinical, epidemiologic, and laboratory criteria. Case finding was done through electronic postings (email and website postings), faxes, and mass media. Active surveillance of persons in

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contact with infected persons or animals included self-recorded diaries of signs and symptoms for 21 days postexposure or daily telephone assessments by local health department personnel. Data were summarized at the Wisconsin Division of Public Health (WDPH).

Willing pet store employees were given a standardized questionnaire to assess prairie dog contact and were offered serologic testing. Affected animal distributors were interviewed about work roles and animal care.

Veterinary Staff Cohort Study

The eligible cohort was defined as all persons, regardless of work roles, employed at any Wisconsin veterinary facility where at least 1 outbreak-associated prairie dog was treated during May 13–27, 2003. Cohort members were defined as those facility employees who participated in the study. Cohort case-patients were defined as cohort members who had laboratory-confirmed MPXV infections, regardless of the presence or absence of specific signs or symptoms. Tissue confirmation required demonstration of MPXV by viral culture, PCR, immunohistochemistry, or electron microscopy. Although cases could not be serologically confirmed by outbreak case definition criteria, cohort members with MPXV infections confirmed by tissue or serologic testing were defined as cohort case-patients. Serologic confirmation required the finding of elevated orthopox immunoglobulin M (IgM) titers in a specimen obtained within 56 days after rash onset or seroconversion in paired acute- and convalescent-phase specimens. The cohort study had no probable or suspected-case definitions and, hence, no probable or suspected cases.

Signs and symptoms surveyed were rash, fever, chills, sweats, headache, joint pain, or lymphadenopathy within 21 days of most recent exposure to an ill prairie dog. Cohort members with a history of vaccinia vaccination or unknown vaccination status and birth date before 1972 were defined as vaccinia-vaccinated.

A standardized questionnaire was used to determine exposure to prairie dogs, general work practices, demographic information, and medical history. Questions to assess contact with prairie dogs during the reception, initial examination, ongoing medical care, and discharge of the prairie dogs had possible answers of yes, no, unknown, or not applicable. Cohort members who did not work within 48 hours after the prairie dog's veterinary visit were excluded from the exposures analysis but included in the remainder of analyses. Questions about general work practices such as sanitizing, hand hygiene (handwashing or cleaning with alcohol gel), and animal bedding changing practices had possible answers of yes, no, unknown, or not applicable; or they used Likert-scale responses of always, usually, sometimes, rarely, never, or not applicable.

WDPH or local health department personnel administered the confidential questionnaire in person or by telephone. Data were entered into Microsoft Office Access 2003 (Microsoft Corp., Redmond, WA, USA) and analyzed using Epi Info version 3.3 (CDC, Atlanta, GA, USA). Likert-scale responses of always and usually were dichotomized from sometimes, rarely, and never. Responses of unknown or not applicable were excluded.

Willing participants provided acute- and convalescent-phase serum specimens, which were tested for nonspecific orthopox virus IgM and IgG levels at the CDC poxvirus laboratory (*20*). Tissue testing was conducted as part of patients' clinical care.

Outbreak-associated prairie dogs treated in Wisconsin veterinary facilities were traced backward and forward. Information was obtained about their illnesses and treatments.

Results

Outbreak Investigation

WDPH received 104 reports of potential human MPXV infections. Of these, 27 represented case-defined illnesses: 19 (70%) confirmed, 5 (19%) probable, and 3 (11%) suspected. Illness onsets occurred during May 15–June 13, 2003 (Figure 1). Based on date of first exposure, the median incubation period was 12 days (range 1–41 days). Median age of case-patients was 28 years (range 3–48 years), and 18 (67%) were female. Patients resided in 5 Wisconsin counties: Milwaukee (n = 14), Waukesha (n = 8), Clark (n = 3), Jefferson (n = 1), and Washington (n = 1). Among confirmed case-patients, those positive by test method were distributed as follows: PCR, 15 (79%); immunohistochemistry, 12 (63%); virus culture, 9 (47%); and electron microscopy, 4 (21%).

Signs and symptoms reported by ≥80% of case-patients were rash, headache, sweats, and fever. Those reported by 60%–70% of case-patients were chills, sore throat, cough, or lymphadenopathy. All other signs and symptoms were reported by ≤23% of case-patients. No statistically significant differences in signs and symptoms were reported between confirmed and probable or suspected case-patients. Five (19%) patients were hospitalized; none died.

In terms of exposure settings, 12 (44%) cases, including 10 confirmed, occurred in staff of veterinary facilities where ill prairie dogs had received care (Figure 2). Other cases occurred in 6 members of households with prairie dogs, 4 pet store visitors, 2 pet store employees, 2 animal distributors, and 1 visitor to a household with prairie dogs. No known cases occurred in healthcare workers who treated patients or in laboratory workers who handled specimens.

Symptom diaries were completed by 258 persons, including 28 pet store employees and 7 veterinary staff. Lo-

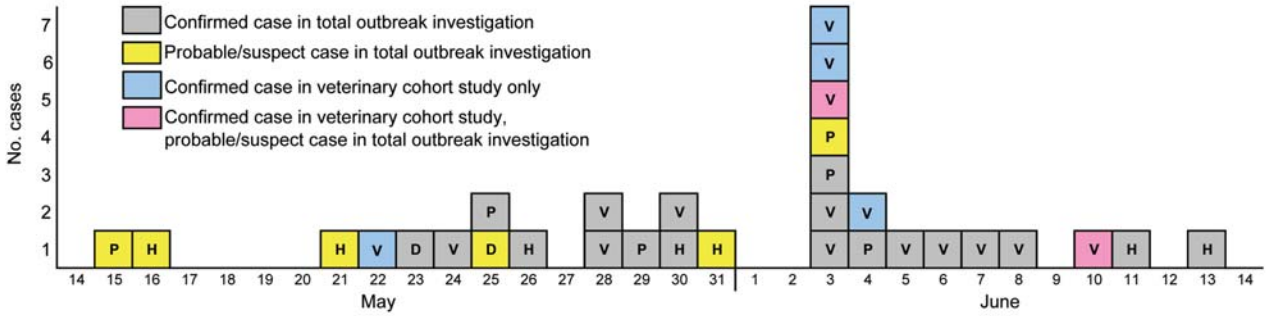

Figure 1. Reported dates of illness onset for persons with monkeypox virus infection. Data from the outbreak investigation and the veterinary facility cohort study, by exposure classification and case status, Wisconsin, 2003. One veterinary cohort case-patient is not included in this figure because of unknown date of illness onset. P, pet store employee or visitor; H, household contact; V, veterinary facility staff; D, animal distributor.

cal health department personnel monitored 243 other persons by telephone, including 77 veterinary staff. Among 501 persons followed up, 10 (2%) experienced illness; all 10 were veterinary staff.

Two pet stores had received outbreak-associated prairie dogs. Of 28 employees (26 from store A, 2 from store B), 19 (68%) completed the questionnaire: 11 had handled prairie dogs, 9 had fed prairie dogs, 2 had been bitten by a prairie dog, and 2 had been scratched by a prairie dog. One store A employee had a confirmed case, and 1 store B employee had a suspected case; both had handled ill prairie dogs. The store A employee had a tissue diagnosis of MPXV confirmed by viral culture and PCR and was positive for orthopox IgM and IgG antibodies in acute- and convalescent-phase serum specimens. The store B employee had prior vaccinia vaccination; a convalescent-phase serum specimen was negative for orthopox IgM and positive for IgG. Approximately 2 months after the last prairie dog exposure, serum specimens were obtained from 12 noncase pet store employees, 4 of whom had handled ill prairie dogs. All 12 had negative orthopox IgM antibody results.

Two Wisconsin exotic animal distributors (distributors 2 and 3, a married couple) distributed outbreak-associated prairie dogs and housed animals in their home. Distributor 2 had a confirmed case of MPXV infection, distributor 3 had a suspected case, and an immunocompromised household member who had no direct animal contact had a confirmed case.

Veterinary Staff Cohort Study

Four veterinary facilities had treated 3 outbreak-associated prairie dogs. These facilities employed 81 (range 3–59) persons during the outbreak; 74 (91%) participated in the cohort study (cohort members), and 44 (54%) participated in the serosurvey. Table 1 summarizes demographic characteristics of cohort members, serosurvey participants, and cohort case-patients. At least 1 veterinarian from each facility was a cohort case-patient. Among 17 cohort case-patients, 8 (47%) had tissue and serologically confirmed cases, 7 (41%) had serologic confirmation only, and 2 (12%) had tissue confirmation only. The 17 cohort case-patients included all 10 veterinary staff with confirmed cases and 2 with probable or suspected cases (previously mentioned in the overall outbreak investigation). Five serologically confirmed cohort case-patients did not meet the definition of an overall outbreak case-patient.

Fever, sweats, chills, rash, lymphadenopathy, and headache were each associated ($p<0.001$) with confirmed MPXV infection (Table 2). Among cohort case-patients, 15 (88%) had multiple signs and symptoms and 2 (12%) had only 1 sign or symptom (headache and a nonvesicular, nonpustular rash of unknown onset date, respectively). Severe keratitis required corneal transplantation for 1 cohort case-patient, and a miscarriage occurred at 12 weeks of gestation. Two cohort case-patients did not experience a rash.

By using the number of cohort members from each facility as the denominator, we calculated veterinary facility attack rates as follows: facility 1, 25%; facility 2, 67%; facility 3, 7%; and facility 4, 25%. The attack rate among cohort members for all 4 facilities combined was 23%. All cohort case-patients had been in the veterinary facility within 48 hours of the prairie dog's visit. The only factor protective against MPXV infection (Table 2) was never having handled an ill prairie dog ($p = 0.004$). Having a job involving direct animal care (e.g., veterinarian, veterinary technician, or veterinary assistant) was associated with having a confirmed case ($p = 0.002$). Four types of exposures were associated with having a confirmed case: participating in an initial ($p = 0.004$) or follow-up ($p = 0.04$) examination of an ill prairie dog, caring for an animal within 6 feet of the ill prairie dog ($p = 0.03$), and feeding the ill prairie dog ($p = 0.002$). Vaccinia vaccination status did not differ between those who performed at least 1 of these 4 high-risk activities and those who did not ($p = 0.9$). All cohort case-patients reported having practiced hand hygiene after examining or feeding the ill prairie dog.

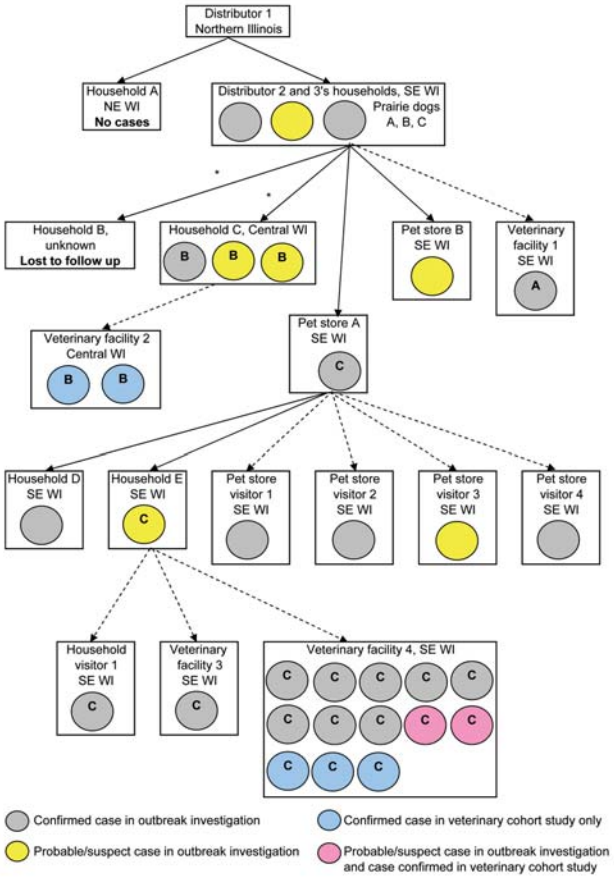

Figure 2. Cases of human monkeypox virus infection. Data from the outbreak investigation and veterinary facility cohort study, by exposure setting and case status, Wisconsin, 2003. A, exposure to prairie dog A; B, exposure to prairie dog B; C, exposure to prairie dog C. Exposure = direct contact or same-room exposure. *Prairie dog sold at swap meet. NE, northeastern; WI, Wisconsin; SE, southeastern; solid arrows, prairie dog sale and exposure; dashed arrows, prairie dog exposure only.

Gloves had been used by cohort case-patients during the following activities: 2 (40%) initial examination, 3 (60%) follow-up examination, and 3 (75%) feeding an ill prairie dog. No cohort case-patients reported having used surgical masks, goggles, or face shields during these high-risk activities. Four cohort case-patients had fed a prairie dog on 8 occasions: placed food in the cage without touching the prairie dog (1×), hand fed prairie dog by syringe (3×), placed food directly in the prairie dog's mouth (3×), or fed through gastric tube (1×). Although having spent ≥30 minutes handling the prairie dog approached significance ($p = 0.09$), 7 (41%) cohort case-patients reported never having handled a prairie dog. All of these 7 cases resulting from indirect exposure occurred in employees of facility 4. Five of these 7 case-patients reported having been within 3 feet of prairie dog C, 1 reported having been in the same room as prairie dog C but not within 3 feet, and 1 reported not being in facility 4 while prairie dog C was there but being there within 48 hours of its death. Multivariate analysis was not possible because of the small number of cohort members with each type of exposure.

Using antihistamines during the prairie dog visit ($p = 0.04$) was associated with being a cohort case-patient (Table 2). Antihistamine use was considered a possible surrogate for hand-to-face contact because users of antihistamines generally have allergies or rhinorrhea and likely touch their eyes or nose frequently. No other personal medical history was associated with illness. Previous vaccinia vaccination ($p = 0.95$) was not protective against MPXV infection. Few cohort members reported immunosuppressive medication use ($n = 3$), immunosuppressive illness ($n = 2$), or being pregnant ($n = 2$).

No general work practice was a protective or risk factor for being a cohort case-patient. General work practices were not outbreak specific and were used to assess overall risk for communicable disease transmission. Several cohort members reported hand-to-mouth activities (eating, drinking, chewing gum, or applying lip products) in animal care areas (Table 3). Only 12% who cleaned ill animals' cages reported having used gloves during this task. Most (92%–93%) cohort members reported cleaning their hands before eating at work and after ill animal contact.

The 44 serosurvey participants included 9 of 10 patients with tissue-confirmed cases and 35 of 64 persons without tissue-confirmed cases ($p = 0.04$). Cohort members with direct animal care jobs were not more likely ($p = 0.19$) than those without such jobs to have participated in the serosurvey. MPXV infection was serologically confirmed for 13 (65%) persons who provided paired serum specimens and 2 (10%) who provided only acute-phase serum specimens. No evidence of asymptomatic seroconversion was found. Among serosurvey participants, only feeding a prairie dog was statistically associated ($p = 0.02$) with having a confirmed case of illness, and no personal medical history factors were associated with illness. A history of vaccinia vaccination was not protective against MPXV infection ($p = 0.35$). Nineteen (43%) serosurvey participants had been vaccinated. Of these, 6 (32%) had MPXV infection; 5 had multiple signs or symptoms; and 1 had only a nonpustular, nonvesicular rash. Four (67%) of the vaccinia-vaccinated serosurvey participants with confirmed cases had serologic confirmation only (no tissue confirmation was attempted), and 1 (17%) had both tissue and serologic confirmation of illness. Two serosurvey participants (A and B) with confirmed cases and previous vaccinia vaccination had no acute elevation of IgM. Participant A had symptomatic illness confirmed by IgG seroconversion and multiple high-risk exposures, including participation in 2 prairie dog examinations and having provided care to an animal within

Table 1. Demographic characteristics of veterinary facility cohort members during outbreak of monkeypox virus infections, Wisconsin, 2003

Demographic variable*	All cohort members (n = 74), no. (%)	Serosurvey participants only (n = 44), no. (%)	Cohort case-patients only (n = 17), no. (%)
Sex			
Female	56 (76)	34 (77)	13 (76)
Male	18 (24)	10 (23)	4 (24)
Race			
White	73 (99)	44 (100)	17 (100)
Unknown	1 (1)	0	0
Ethnicity			
Not Hispanic	67 (90)	40 (91)	16 (94)
Hispanic	6 (8)	4 (9)	1 (6)
Unknown	1 (1)	0	0
Job title			
Veterinarian	12 (23)	9 (20)	6 (35)
Veterinary technician	18 (24)	14 (32)	6 (35)
Veterinary assistant	18 (24)	11 (25)	5 (29)
Receptionist	14 (19)	6 (14)	0)
Clinic manager	4 (5)	4 (9)	0
Other†	3 (4)	0	0
No. employees			
Facility 1 (n = 4)	4 (5)	3 (7)	1 (6)
Facility 2 (n = 3)	3 (4)	3 (7)	2 (12)
Facility 3 (n = 15)	14 (19)	5 (11)	1 (6)
Facility 4 (n = 59)	53 (72)	33 (75)	13 (76)

*Ages, median (range), y: all cohort members, 31 (17–57); serosurvey participants, 31 (20–57); cohort case-patients, 31 (23–47).
†Custodial and information services staff.

6 feet of the prairie dog. Participant A's IgM levels were not elevated at 15, 36, and 50 days after exposure. Participant B had symptomatic, pathologically confirmed illness and multiple high-risk exposures, including participation in 4 prairie dog examinations, having fed the prairie dog, and having provided care to an animal within 6 feet of the prairie dog. Participant B's IgM results were not elevated, and IgG results were positive without a boost in titer at 16 and 157 days after exposure. Participant B had a history of bone marrow ablation and an allogenic bone marrow transplant.

An Illinois animal distributor (distributor 1) obtained prairie dogs from a Texas distributor (C. Austin, pers. comm.). During April–May, 2003, distributor 1 housed ≈200 prairie dogs with African rodents that had been purchased on April 21 and subsequently implicated in MPXV introduction (*2*,*6*,*7*). Distributor 2 purchased 39 prairie dogs, including prairie dogs A, B, and C, from distributor 1 and transported them to Wisconsin during April 15–May 17 (*1*,*4*). Prairie dog A remained in the custody of distributor 2 until it was taken to facility 1 (Figure 2) for 10 minutes for carbon dioxide chamber euthanasia on May 13, 2003. Prairie dog B was sold at a swap meet on May 11; became ill with conjunctivitis, lymphadenopathy, and papular skin lesions on May 13; was examined at facility 2 for 10–30 minutes on May 15; and died on May 20. Prairie dog C was sold to pet store A on May 5 and sold to a customer on May 17. On May 19, prairie dog C had illness onset with conjunctivitis, skin lesions, and respiratory disease. Prairie dog C was examined in facility 3 for 20–25 minutes on May 22 and hospitalized at facility 4 from May 25 until its death on May 27. Facility 4 staff provided extensive treatment including repeated examinations; hand feeding; eye discharge removal; and oral, subcutaneous and nebulized treatments. Sixteen (59%) outbreak cases and 14 (82%) veterinary cohort cases were associated with prairie dog C.

Discussion

The 2003 outbreak of MPXV infections affected Wisconsin residents who had been exposed in multiple settings; however, 59% of cases occurred among occupationally exposed persons. Our cohort study demonstrates that veterinary staff were particularly at risk (23% attack rate). Pet store employees were at lower risk (7% attack rate). Infected prairie dogs were probably more ill and shedding more virus while in veterinary facilities than in pet stores, which would account for more observed infections among veterinary staff. Both Wisconsin distributors of ill prairie dogs became ill. The preponderance of occupationally acquired cases was unique to Wisconsin during this outbreak. Among other involved states, 1 veterinarian from Indiana had a suspected case, and cases occurred in 2 employees of distributor 1 (*7*,*21*,*22*).

Most outbreak cases (59%) and veterinary cohort cases (82%) were associated with exposure to prairie dog C. We found no intrinsic differences in the monkeypox infection of prairie dog C compared with that of prairie dogs A or B

and no explanation for this association, although length and type of exposures in facility 4 were likely accountable. Facility 4 had many more employees than the other facilities, and prairie dog C was hospitalized there for a relatively prolonged period (3 days) and received extensive treatments there. In addition, the fact that 7 (54%) facility 4 cohort case-patients reported never having handled prairie dog C indicates that other transmission modes (e.g., fomites, aerosols) could have contributed to that facility's large number of cases. Nebulization treatments, which prairie dog C received ≈4× at facility 4, could have exposed employees to MPXV. Although nebulization was performed in an enclosed plastic chamber, the nature of the treatment would foster aerosolization, coughing, and possibly mobilization of respiratory secretions with which employees could have unknowingly come into contact.

Although our Wisconsin investigation showed no definitive evidence of human-to-human transmission of MPXV, which concurs with other states' findings during this multistate outbreak (*6*,*7*,*21*,*23*), such transmission remains a possibility. Because persons who had had no direct contact with ill animals became ill with MPXV infection, person-to-person transmission within veterinary facilities might have occurred. However, because of the lack of personal protective equipment use among the cohort members and the finding of MPXV in ill prairie dog's urine and feces

Table 2. Medical data for veterinary facility cohort members during outbreak of monkeypox virus infections, by case status, Wisconsin, 2003*

Patient data	Cohort case-patients, no./total† (%)	Cohort members without confirmed case, no./total† (%)	p value‡	RR	95% CI
Signs and symptoms					
Rash	15/17 (88)	3/57 (5)	<0.001	23.3	5.9–92.4
Fever	12/16 (75)	5/57 (9)	<0.001	9.9	3.7–26.7
Chills	14/17 (82)	4/57 (7)	<0.001	14.5	4.7–44.9
Sweats	14/17 (82)	8/57 (14)	<0.001	11.0	3.5–34.6
Headache	13/17 (76)	7/57 (12)	<0.001	8.8	3.2– 23.8
Joint pain	6/17 (35)	7/56 (13)	0.06	2.5	1.1–5.6
Lymphadenopathy	11/16 (69)	2/57 (4)	<0.001	10.2	4.3–24.3
Exposures to ill prairie dogs					
Was in clinic within 48 h after prairie dog visit	17/17 (100)	50/57 (88)	0.19	UND	NA
Admitted prairie dog to facility	1/17 (6)	6/44 (14)	0.66	0.5	0.1–3.1
Involved in initial evaluation of prairie dog	5/16 (31)	1/44 (2)	0.004	4.1	2.2–7.7
Cared for animal within 6 feet of prairie dog	9/12 (75)	12/31 (39)	0.03§	3.1	1.0–10.0
Gave oral antimicrobial drugs	3/12 (25)	4/40 (10)	0.33	2.1	0.8–6.0
Gave subcutaneous fluids	4/12 (33)	5/41 (12)	0.18	2.4	0.9–6.4
Gave subcutaneous antimicrobial drugs	1/11 (9)	3/41 (7)	1.00	1.2	0.2–7.2
Gave nebulized therapy	3/12 (25)	4/41 (10)	0.18	2.2	0.8–6.2
Involved in follow-up examination	5/12 (42)	5/41 (12)	0.04	3.1	1.2–7.7
Took radiographs of prairie dog	1/11 (9)	1/41 (2)	0.38	2.5	0.6–11.1
Fed prairie dog	4/12 (33)	0/41 (0)	0.002	6.1	3.3–11.6
Cleaned cage of prairie dog	12/12 (100)	36/43 (84)	0.32	UND	NA
Cleaned discharge from prairie dog's eyes	6/15 (40)	7/41 (17)	0.09	2.2	1.0–5.0
Examined any animal on surface used to examine prairie dog within past 24 h	7/11 (64)	9/30 (30)	0.07	2.7	1.0–7.9
Spent >30 min handling prairie dog	4/17 (24)	4/53 (8)	0.09	2.4	1.0–5.6
Never handled prairie dog	7/17 (41)	43/53 (81)	0.004	0.28	0.1–0.6
Spent >30 min within 3 feet of prairie dog	7/17 (41)	14/53 (26)	0.25§	1.6	0.7–3.7
Job involved direct animal care	17/17 (100)	36/57 (63)	0.002	UND	NA
Medical history					
Vaccinia vaccination	7/17 (41)	23/57 (40)	0.95§	1.0	0.4–2.4
Atopic dermatitis	1/17 (6)	4/57 (7)	1.0	0.9	0.1–5.2
Seasonal allergies	7/17 (41)	21/57 (37)	0.75§	1.2	0.5–2.7
Open sores at time of prairie dog visit	5/17 (29)	9/57 (16)	0.28	1.8	0.8–4.3
Upper respiratory infection at time of prairie dog visit	3/17 (18)	3/57 (5)	0.13	2.4	1.0–6.1
Antihistamine use at time of prairie dog visit	4/17 (24)	3/57 (5)	0.04	3.0	1.3–6.6

*RR, relative risk; CI, confidence interval; UND, undeterminable; NA, not accurate.
†Denominators vary according to total no. persons with work roles appropriate to the exposure; e.g., a receptionist would not be expected to administer subcutaneous fluids.
‡Fisher exact 2-tail test unless otherwise indicated.
§Mantel-Haenszel test.

Table 3. General work practices of 74 veterinary facility cohort members during outbreak of monkeypox virus infections, Wisconsin, 2003*

Work practice	No. (%)
Sanitizes examination table	44 (81)
Sanitizes examination room countertops	32 (59)
Eats in work break room	59 (82)
Eats where animals are treated or housed	1 (1)
Drinks where animals are treated or housed	10 (14)
Chews gum where animals are treated or housed	11 (15)
Applies lip products where animals are treated or housed	6 (8)
When cleaning cages, agitates bedding enough to aerosolize material in cage	5 (10)
Wears gloves when cleaning ill animals' cages	6 (12)
Does animal laundry at work	31 (58)
Cleans hands after contact with ill animals	65 (93)
Cleans hands before eating at work	67 (92)
Cleans hands when leaves work	57 (77)
Changes out of work shoes before leaving work	8 (11)
Changes out of work clothes before leaving work	7 (11)
Washes work clothes at home	70 (95)
Washes laboratory coat at home	14 (50)
Washes work clothes between work shifts	67 (93)
Washes laboratory coat between work shifts	15 (50)

*The denominators vary according to the number of cohort members who perform a given task.

(*21*), environmental exposure may well account for these cases.

The substantial amount of illness among veterinary staff underscores the importance of infection-control practices in veterinary settings. Cohort case-patients frequently did not use personal protective equipment during high-risk activities (e.g., examining or feeding ill prairie dogs). Furthermore, cohort members reported general work practices that foster hand-to-mouth activities in animal care areas. Few (12%) cohort members reported having used gloves when cleaning ill animals' cages, a task that can contaminate staff hands with animal dander, urine, and fecal matter. We cannot determine whether infection-control guidelines would have prevented MPXV infections among veterinary staff, but use of personal protective equipment might have limited viral transmission. The National Association of State Public Health Veterinarians recently released the Veterinary Standard Precautions Compendium (*24*), the first guidelines to describe standard infection-control practices for veterinary facilities. Use of these guidelines should be encouraged.

In contrast with results from a previously published study (*25*), results of our cohort study do not support the conclusion that prior vaccinia vaccination protected against MPXV infection in this outbreak. Hammarlund et al. found that 3 (37%) of 8 vaccinia-vaccinated persons in this outbreak had asymptomatic MPXV infections and surmised that they had long-term immunity against MPXV infection (*25*). Our cohort study showed that previous vaccinia vaccination did not protect against MPXV infection; all previously vaccinated serosurvey participants with positive serologic results had at least 1 sign or symptom of MPXV infection. The more systematic inclusion and analysis of exposed persons within our cohort, compared with the cohort-series approach of Hammarlund et al., may account for the difference in this finding (*26*).

Our case definition for the cohort study differed from that of the overall outbreak investigation. Among veterinary staff, the 2 definitions resulted in different case numbers: the outbreak case definition resulted in 10 confirmed and 2 probable or suspected cases; the cohort study case definition resulted in 17 confirmed cases. Serologic results were not a confirming criterion in the outbreak case definition because their results were not validated at the time and because serologic specimens were not systematically gathered throughout the multistate outbreak.

For unknown reasons, 2 cohort case-patients had no elevation of orthopox IgM. Although the IgM response might have been missed, this is unlikely given the timing of specimen collections. Also, previous vaccinia vaccination might have altered the immune response to the MPXV infection. It is also possible that participant B's past medical history might have affected the immune response to this infection.

Our study has several potential limitations. Although 91% of employees at the 4 affected veterinary facilities participated in the cohort study, only 54% participated in the serosurvey. Persons with tissue-confirmed illness were more likely than persons without such illness to have participated in the serosurvey. These factors might have resulted in an underestimation of overall cases and limited the detection of asymptomatic seroconversion. Recall bias, which might have overestimated the association between prairie dog contact and illness, was likely limited by relatively brief intervals between exposures and data collection. Finally, statistical analysis beyond univariate analysis was

limited because of the small number of cohort members involved in each of the high-risk prairie dog exposures.

Our investigation and cohort study demonstrate that occupational exposure, especially among veterinary staff, was a critical factor during this outbreak. This outbreak highlights the importance of standard infection-control guidelines developed for veterinary settings and the need to encourage their use.

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Dr Croft is a physician and medical epidemiologist at the University of Wisconsin School of Medicine and Public Health, Section of Allergy, Pulmonary and Critical Care Medicine. Her research interests focus on lung cancer prevention and detection, communicable disease epidemiology, and healthcare disparities.

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Venezuelan Equine Encephalitis Virus Infection of Cotton Rats

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Venezuelan equine encephalitis virus (VEEV) is an emerging pathogen of equids and humans, but infection of its rodent reservoir hosts has received little study. To determine whether responses to infection vary among geographic populations, we inoculated 3 populations of cotton rats with 2 enzootic VEEV strains (Co97-0054 [enzootic ID subtype] and 68U201 [enzootic IE subtype]). The 3 populations were offspring from wild-caught cotton rats collected in a VEE-enzootic area of south Florida, USA; wild-caught cotton rats from a non–VEE-enzootic area of Texas, USA; and commercially available (Harlan) colony-reared cotton rats from a non–VEE-enzootic region. Although each population had similar early viremia titers, no detectable disease developed in the VEE-sympatric Florida animals, but severe disease and death affected the Texas and Harlan animals. Our findings suggest that the geographic origins of cotton rats are important determinants of the outcome of VEE infection and reservoir potential of these rodents.

Vertebrate reservoir hosts play an important role in maintenance and dissemination of zoonotic pathogens. For arthropodborne viruses (arboviruses), infected hosts generally show little or no disease, which presumably reflects long-term selection for host resistance and possibly virus attenuation (*1*,*2*). Understanding how pathogens affect their reservoir hosts as well as how the reservoir affects fitness and replication of the pathogen could enable better prediction of emergence, reemergence, or extinction of zoonotic pathogens in response to environmental changes. For example, changes in a reservoir host's habitats and ecology, due to anthropogenic or natural causes, may affect pathogen transmission to humans and domestic animals. A better understanding of reservoir biology and of pathogen-reservoir interactions, particularly their mechanisms of disease resistance, could also facilitate the development of treatment and control strategies for humans and domestic animals (*3*,*4*).

Venezuelan equine encephalitis virus (VEEV), a member of the family *Togaviridae*, genus *Alphavirus* (*5*), was first isolated and characterized in 1938 (*6*,*7*) and affects humans and equids in the Americas (*8*,*9*). VEEV strains are classified into 2 epidemiologic groups: enzootic and epizootic. Enzootic strains (subtype I, varieties D and E, as well as related species in the VEE complex comprising subtypes II–VI) have been regularly isolated in lowland tropical forests and swamps in Florida, Mexico, and Central and South America. These enzootic viruses generally circulate between *Culex (Melanoconion)* spp. mosquito vectors and rodent reservoirs and are usually avirulent for and incapable of amplification in equids (*8*). In contrast, epizootic (and epidemic) VEEV strains (subtype I, variety AB and variety C), have been responsible for all major equine outbreaks that have involved other mosquito vectors, primarily *Aedes* and *Psorophora* spp. Epizootics and epidemics have occurred from southern North America to northern South America, and the VEEV strains involved have caused debilitating neurologic disease with high fatality rates in equids (*9*–*11*). In humans, who are tangential hosts during endemic and epizootic cycles, severe febrile illness can become life threatening. Although <1% of infected humans die, permanent neurologic sequelae can occur in survivors, particularly young children (*8*,*12*,*13*).

Serosurveys have found VEEV antibodies in many species of small mammals (*14*–*18*). However, spiny rats (*Proechimys* spp., family Echimyidae*)* and cotton rats (*Sigmodon* spp., family Cricetidae) have been most often implicated as principal reservoir hosts for enzootic strains, based on seroprevalence and experimental infections demonstrating viremia adequate in titer and duration to infect enzootic

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mosquito vectors (*19*–*22*). Their geographic distributions are different, but overlapping: *Proechimys* spp. are found mainly in Panama, northern Peru, Bolivia, Paraguay, and Brazil; *Sigmodon* spp. are found mainly from southern North America to northern parts of Venezuela, Colombia, and Peru (*23*).

Studies supporting the role of cotton rats as reservoir hosts for enzootic VEEV have investigated viremia and antibody responses as well as horizontal transmission in laboratory settings (*19*–*21*,*24*,*25*). Howard et al. reported illness and death in cotton rats after infection with a Texas epizootic subtype IB strain (*21*). The cause of death was linked more to experimental manipulation than to virus infection. Another study that examined clinical and histopathologic manifestations after infection of rats with Everglades virus (EVEV; subtype II in the VEE complex) reported that although viremia developed and the virus replicated in a wide variety of organs, only 2% died (*25*).

The southern United States has 12 native subspecies of cotton rats (*26*), which differ by as much as 5% in their cytochrome b DNA sequence (*27*). To determine whether responses to infection vary among these geographic populations, we studied infection with 2 different subtypes of enzootic VEEV in 3 populations of cotton rats.

Materials and Methods

Animals

Three subspecies of *Sigmodon hispidus* (cotton rat) were used in this study: the Harlan colony, the Texas population, and the Florida population. The Harlan colony consisted of 6- to 8-week-old female rats purchased from Harlan (Indianapolis, IN, USA) from an established colony. Because the exact geographic origin was unknown, cytochrome B mitochondrial gene sequences were amplified by PCR and compared phylogenetically with those from cotton rats from various locations in North America (*27*). The sequences from the Harlan colony grouped with those of animals collected in east Texas, Louisiana, and Tennessee but were outside of the clade from southern Florida (data not shown), which indicated that these rats originated from a nonenzootic region. (Florida is the only VEE complex alphavirus–enzootic region in the United States, aside from the Rocky Mountains, which are outside of *Sigmodon* distribution [*8*,*23*]). The Texas population consisted of 4- to 12-month-old wild-caught male and female *S. hispidus berlandieri* trapped in Galveston Island State Park, Texas (29.27°N, 94.83°W) (*25*). The Florida population consisted of 3- to 21-week-old male and female cotton rats (*S. hispidus spadicipygus)* trapped in southern Florida (*25*) and used to rear F1 rats for experimental studies. Before inoculation, all rats were tested for antibodies against VEEV, EVEV, and Eastern equine encephalitis virus (EEEV) and acclimated for 3 days in a Biosafety Level 3 animal facility. All experiments included ≥2 animals as negative controls. All studies were approved by the University of Texas Medical Branch Animal Care and Use Committee.

Viruses and Infections

VEEV strain Co97-0054 (enzootic ID subtype), isolated in Colombia in 1997 from a sentinel hamster, and strain 68U201 (enzootic IE), isolated in Guatemala from a sentinel hamster and derived from a cDNA clone (*28*), were used for experimental infections. These strains were selected because they had low passage histories and represent the 2 major enzootic VEEV subtypes; strain Co97-0054 has also been used for experimental infection of spiny rats (*22*). Cotton rats were inoculated subcutaneously in the left footpad or left thigh with 3–4 $\log_{10}$ PFU of virus, a dose consistent with the maximum amount of VEEV in mosquito saliva (*29*). To determine whether an increase in virus inoculum could change the outcome of the disease, 4 Florida rats were also inoculated with 5–6 $\log_{10}$ PFU of VEEV strain 68U201. All animals were observed for signs of illness once a day for 15 days.

To determine the neurovirulence of VEEV in the Florida population, 3 rats (2 months of age) were inoculated intracranially with 3 $\log_{10}$ PFU/mL of strain 68U201. Blood samples were collected 24 h after inoculation, and the rats were observed for signs of disease.

Viremia Assays

Blood samples were collected from the retroorbital sinus for ≤10 days after inoculation. Serum samples were diluted 1:10 in Eagle minimal essential medium supplemented with 20% fetal bovine serum, gentamicin, and L-glutamine and stored at –80°C. Viremia titers were determined by plaque assay with Vero cells (*30*).

Antibody Assays

Plaque reduction (80%) neutralization (PRNT) and hemagglutination inhibition (HI) tests were performed (*30*). To detect specific VEEV immunoglobulin M (IgM) antibodies, an IgM-capture ELISA was performed (*31*). Briefly, microplates were coated with anti-rat IgM, diluted 1:500 in carbonate-bicarbonate buffer pH 9.6 (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), and incubated at 4ºC for at least 16 h. Subsequently, the following were sequentially added: test serum, mouse immune ascitic fluid prepared against VEEV antigens, anti-mouse conjugate (Kirkegaard and Perry), and ABTS (2,2′-azinobis[3-ethylbenzthiazoline-6-sulphonic acid])–peroxidase substrate (Kirkegaard and Perry). Test serum samples were diluted at 1:40 in 0.5% bovine serum albumin in phosphate-buffered saline at pH 7.4, and the plates were read by using a spectrophotometer with a 405-nm wavelength filter. The

cut-off value was calculated as the mean optical density $(OD)_{405\ nm}$ of negative control samples plus 3 standard deviations, or 0.200. Linear regression, the Student *t* test, and analysis of variance were used to analyze data.

Results

Clinical Responses and Survival

Inoculation of the Florida rats with 3 $\log_{10}$ PFU of VEEV strain 68U201 (IE) or Co97-0054 (ID) resulted in no detectable signs of illness and survival rates of 100% and 87.5%, respectively (Figure 1). One Florida rat inoculated with strain Co97-0054 died on day 10 postinoculation without exhibiting any signs of illness. These findings contrasted with the results of VEEV infections of the Texas and Harlan populations. Although the Harlan rats were inoculated with only the subtype ID strain, signs of severe illness developed in all of the Harlan and Texas rats beginning on day 5. Signs included ruffled coats, lack of grooming, lethargy, and for many, signs of encephalitis (incoordination and instability when walking and erratic movements of the head and limbs), dehydration (measured by lack of skin turgor), and anorexia. Most animals that died before day 5 postinoculation showed no prior signs of illness. The average survival time for the Texas population was 6.8 days after inoculation with the subtype ID strain and 8.2 days with the IE strain; for the Harlan colony, it was 5 days after inoculation with the subtype ID VEEV. None of the animals that survived past day 15 died. The 2 sham-inoculated and the 2 noninoculated rats survived without signs of disease.

Neurovirulence in Florida Cotton Rats

To determine whether the absence of disease in the Florida population was due to the inability of the virus to penetrate the central nervous system, 3 rats were inoculated intracranially with 3 $\log_{10}$ PFU/mL of subtype IE VEEV. Viremia titers at 24 h postinoculation were 6.3, 6.2, and 6.8 $\log_{10}$ PFU/mL (mean 6.5). By day 3 postinoculation, all of these rats started showing signs of illness, including ruffling of the fur and lack of movement. By day 9 postinoculation, 1 rat was dead and the other 2 exhibited instability and difficulty in walking, uncoordinated and erratic movements of the head and limbs, dehydration, and anorexia; these animals were euthanized because of the severity of disease. Histopathologic examination of the brains showed clear signs of encephalitis, focal meningoencephalitis (Figure 2, panels A, B) and associated perivascular mononuclear cell infiltration (Figure 2, panel C), and neurophagia, which led to the conclusion that the cause of death was from the viral infection and not from the injection or manipulation of the animals.

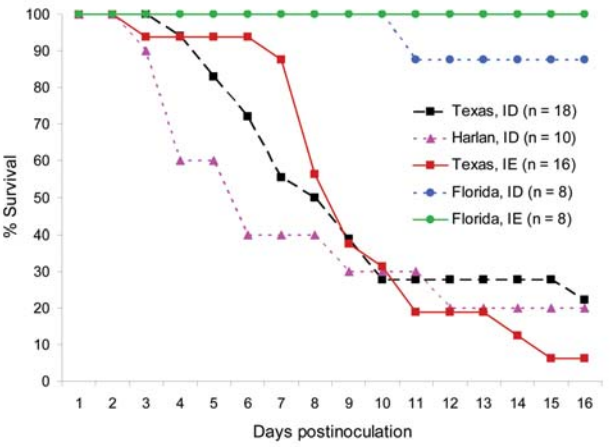

Figure 1. Survival of cotton rats from Florida, Texas, and Harlan after subcutaneous inoculation with 3 $\log_{10}$ PFU of enzootic Venezuelan equine encephalitis virus (subtypes IE and ID).

Dose Dependence

To determine whether the Florida population's apparent resistance to VEE after peripheral infection was dose-dependent, 8 additional animals were inoculated with 5 or 6 $\log_{10}$ PFU (4 animals per dose). In each population, 3 (75%) of the rats survived infection (data not shown). One rat inoculated with 6 $\log_{10}$ PFU of virus succumbed to disease on day 3, whereas another animal inoculated with 5 $\log_{10}$ PFU of virus died on day 11, which suggests that the dose could have affected disease progression. None of the other inoculated animals showed any clinical signs of illness.

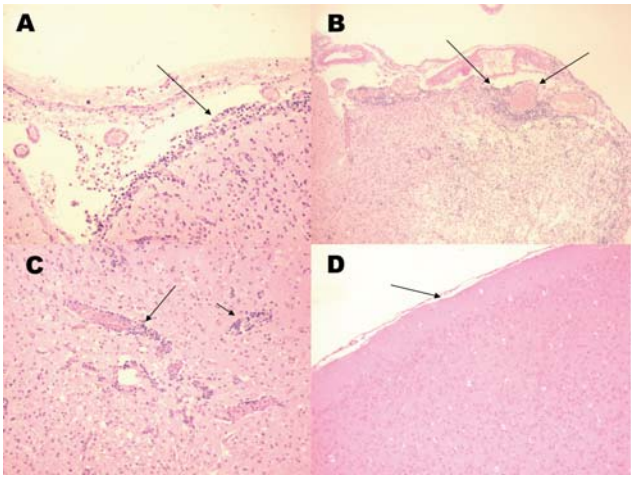

Figure 2. Histologic staining (hematoxylin and eosin) of Florida cotton rat tissues 9 days after intracranial inoculation with 3 $\log_{10}$ PFU of enzootic Venezuelan equine encephalitis virus (subtype IE). A) Inflammation of the meninges (arrows). B) Inflammation of the meninges and dilated blood vessels (arrows). C) Perivascular cuffing of blood vessels (arrow). D) Brain from a noninfected rat. (Magnification ×40.)

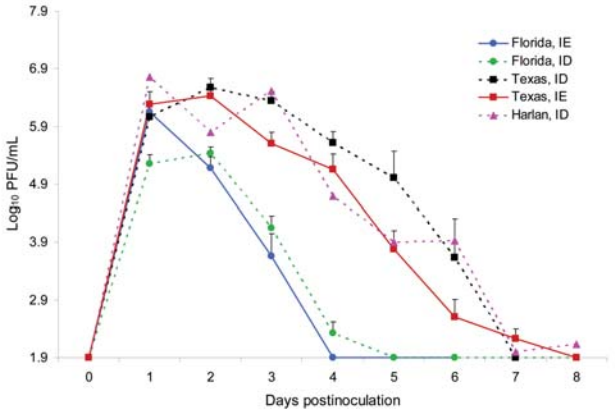

Figure 3. Mean viremia titers of cotton rats from Florida, Texas, and Harlan after subcutaneous inoculation with 3 $\log_{10}$ PFU of enzootic Venezuelan equine encephalitis virus (subtypes IE and ID). Bars indicate standard errors of the means.

Viremia Titers

Viremia profiles for the Florida rats were similar after inoculation (3 $\log_{10}$ PFU) with either subtype ID or IE VEEV ($p>0.05$). Peak viremia titers of 6.2 and 5.4 $\log_{10}$ PFU/mL, respectively, occurred at 24–48 h postinoculation, then became undetectable by days 4–5 postinoculation (Figure 3). The Texas population inoculated with 3–4 $\log_{10}$ PFU of subtype IE virus and 3 $\log_{10}$ PFU of subtype ID VEEV had similar viremia profiles, with peak titers of 6.1 and 6.6 $\log_{10}$ PFU/mL, respectively, at 24–48 h.

We found in the Florida population a correlation between age and peak viremia titers. Younger animals inoculated with the IE virus had higher peak titers on days 1 and 2 postinoculation than did older animals (Figure 4, Table 1). In addition, we observed a significant difference in mean viremia titers on day 2 between 3- and 8-week-old animals and on day 3 between 5- and 8-week-old animals and between 3- and 8-week-old animals ($p<0.05$).

Differences in Virus Titers among Cotton Rat Populations

No differences in viremia profile were observed between the Texas and Harlan rats, for which VEEV infection with subtype ID was generally fatal ($p>0.05$). Viremia in the Texas and Harlan rats peaked between 24 and 48 h postinoculation, with mean titers of ≈6.7 $\log_{10}$ PFU/mL, and was undetectable by day 8 postinoculation. In contrast, significant differences were observed in peak viremia titers between the Florida and Texas rats inoculated with either the ID or IE VEEV subtype ($p<0.05$). In all cases, Texas rats produced higher titers (24 h postinoculation) and had a longer duration of viremia than the Florida rats, in which no disease was apparent (Figure 3, Tables 2, 3). Similar results occurred when Florida and Harlan rats inoculated with subtype ID were compared.

Antibody Responses

To determine whether the difference in disease outcome in Florida versus Texas rats was due to a difference in antibody responses, serum was tested by PRNT. In the Texas population inoculated with subtype IE VEEV, low titers of neutralizing antibodies (NAb) were produced by days 5–6 postinoculation (mean titer 20), and mean titers never exceeded 40 by day 10 (Figure 5, panel A); however, detectable NAb were not produced in all animals that died. In contrast, some Florida rats inoculated with the same virus strain had detectable NAb titers by day 4, and all had detectable titers by day 6. NAb titers were significantly lower in the Texas rats on days 5, 6, and 7 ($p = 0.02$, 0.04, 0.04, respectively).

When Texas rats were inoculated with the subtype ID VEEV strain, even lower NAb titers were produced, despite the development of higher viremia titers during the later stages, compared with those inoculated with subtype IE. Peak NAb titers occurred on day 8 postinoculation (mean 25), and 2 of the surviving animals had no detectable NAb titers (<19) late during infection (day 32). In contrast, in all Florida rats, detectable NAb developed by day 4 and peaked on day 6 (mean 57), when they were significantly higher ($p = 0.004$).

To determine the duration of the antibody response in the Florida population, we measured NAb titers in 2 rats for 7 months postinoculation. Titers peaked at 4 months and then gradually decreased to the detection limit by 7 months, when the experiment was terminated (Figure 5, panel B).

Because NAb were not detected in all infected animals, serum samples were further tested by HI and IgM ELISA. All rats had detectable HI antibodies by days 5–6 postinoculation. Although the titers were relatively low (<200) on

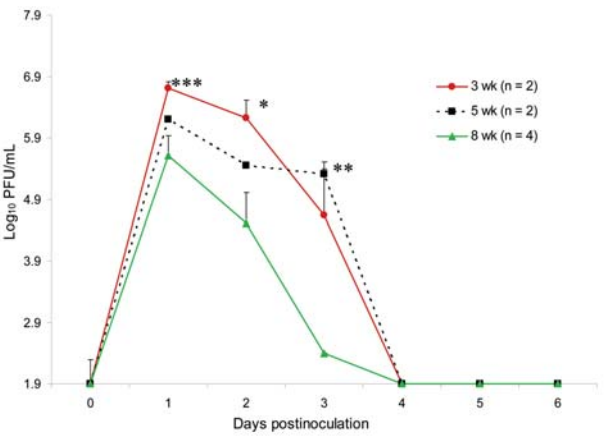

Figure 4. Age-dependent viremia in Florida cotton rats inoculated subcutaneously with 3 $\log_{10}$ PFU of subtype IE Venezuelan equine encephalitis virus. Randomly picked female and male animals aged 3–8 weeks were inoculated subcutaneously with 3 $\log_{10}$ PFU. Significant differences were detected on day 2 postinoculation (*$p = 0.007$) and day 3 (**$p = 0.02$) but not on day 1 (***$p = 0.06$). Bars indicate standard errors of the means.

Table 1. Mean viremia titers of Florida cotton rats inoculated subcutaneously with Venezuelan equine encephalitis virus, subtype IE*

Day postinfection	Age, wk		
	3 (n = 2)	5 (n = 2)	8 (n = 4)
1	6.7	6.2	5.6
2	6.2	5.4	4.5
3	4.6	5.3	2.4
4	1.9	1.9	1.9
5	1.9	1.9	1.9
6	1.9	1.9	1.9

*Titers are expressed as $\log_{10}$ PFU/mL.; 1.9 $\log_{10}$ PFU/mL is the limit of detection of the plaque assay.

day 6, titers increased constantly over the 10 days tested; no differences in titers were noted between VEEV subtype ID or IE infections in the Texas population. As was found by PRNT, HI antibody titers were higher for the Florida population than the Texas population, and titers were higher in animals inoculated with the ID than with the IE VEEV strain (Figure 5, panel C).

Because of volume limitations of daily blood collection, IgM titrations were performed only on blood samples from euthanized rats (2 rats per day per group). Both Florida and Texas populations had similar IgM titers during the first 7 days postinoculation, regardless of the virus used. Mean titers ranged from OD 0.3 on day 4 to OD 0.8 by day 7 in the Texas population and 0.5 in the Florida population (Figure 5, panel D).

In summary, NAb titers were higher in Florida rats inoculated with subtypes ID and IE than in Texas rats inoculated with the same viruses. This finding suggests that these animals may have mounted a more rapid and effective immune response that protected against severe VEE infection.

Discussion

Reservoir Status and Potential

S. hispidus, a main reservoir host of VEE complex alphaviruses, comprises >22 subspecies in North America alone (*26*) that differ by up to 5% in their cytochrome b mitochondrial DNA sequences (*27*). Because some but not all North American populations occur in regions enzootic for VEE complex alphaviruses (e.g., EVEV in Florida), we attempted to better understand the host-VEEV interactions by inoculating 3 different populations with enzootic VEEV strains. Cotton rats from the enzootic area of southern Florida (sympatric with EVEV) responded to VEEV infection as expected: moderate viremia titers, seroconversion by days 4–5 postinoculation coincident with viremia cessation, and few deaths and little detectable disease. This apparently commensal relationship could reflect long-term selection for cotton rat resistance to EVEV in Florida. Although EVEV is a relatively benign virus in laboratory rodents, the ancestral form of EVEV, believed to be a subtype ID VEEV strain from Panama or South America, is more virulent (*32*).

In contrast, rats from 2 nonenzootic locations (the Harlan and Texas populations) had dramatically different outcomes: severe disease often culminated in clinical signs of encephalitis and high mortality rates. This difference in disease and survival was more pronounced than that reported in other studies of VEEV-reservoir host interactions, some of which suggested that cotton rats die because of experimental manipulations or anesthesia rather than from a viral cause (*19–21,24*). Our results indicate that VEEV was the cause of death for most or all of our rats, and signs of encephalitis were consistent with those described in VEEV-infected mice or guinea pigs (*8,33,34*). The rats infected in previous studies had several different geographic origins (Arizona, North Carolina, Florida, and Panama). Of these, only Panama and Florida are enzootic for VEE complex alphaviruses. North Carolina is enzootic for another closely related alphavirus, EEEV, for which birds are thought to be the main reservoirs (*35*). These allopatric rat populations should be reexamined to further test the hypothesis that lack of exposure to VEE complex or other alphaviruses has resulted in no selection for resistance. On the basis of previous susceptibility studies, the peak viremia titers in all infected cotton rats were sufficient to infect known enzootic vector mosquitoes (*36,37*).

Reservoir hosts play an important role in the maintenance and spread of zoonotic viruses. They generally show little or no disease after infection with VEEV and most

Table 2. Mean viremia titers of Florida, Texas, and Harlan cotton rats inoculated subcutaneously with Venezuelan equine encephalitis virus, subtype ID or IE*

Day postinfection	Cotton rat population and virus subtype				
	Florida, IE	Florida, ID	Texas, IE	Texas, ID	Harlan, ID
1	6.1 ± 0.2	5.2 ± 0.2	6.3 ± 0.2	6.1 ± 0.2	6.8 ± 0.9
2	5.2 ± 0.2	5.4 ± 0.1	6.4 ± 0.1	6.6 ± 0.2	5.8 ± 0.9
3	3.7 ± 0.4	4.1 ± 0.2	5.6 ± 0.2	6.3 ± 0.2	6.5 ± 0.5
4	<1.9	2.3 ± 0.2	3.8 ± 0.3	5.6 ± 0.2	4.7 ± 0.8
5	<1.9	<1.9	2.6 ± 0.3	5.0 ± 0.5	3.9 ± 0.1
6	<1.9	<1.9	2.2 ± 0.2	3.6 ± 0.7	3.9 ± 0.2
7	<1.9	<1.9	<1.9	<1.9	2.0 ± 0.0
8	<1.9	<1.9	<1.9	<1.9	2.1 ± 0.3

*Titers are expressed as $\log_{10}$ PFU/mL ± standard error; 1.9 $\log_{10}$ PFU/mL is the limit of detection of the plaque assay.

Table 3. Statistical comparisons (p values) among viremia titers in Texas and Florida cotton rats inoculated with Venezuelan equine encephalitis virus, subtype IE or ID*

Day postinfection	Texas vs. Florida, subtype IE	Texas vs. Florida, subtype ID	Subtype IE vs. ID, Texas	Subtype IE vs. ID, Florida
1	0.123	**0.018**	0.590	0.078
2	**5.0×10^{-4}**	**4.69×10^{-5}**	0.506	0.078
3	**9.75×10^{-7}**	**2.72×10^{-6}**	0.506	0.215
4	**2.82×10^{-10}**	**3.66×10^{-8}**	0.348	0.751
5	**1.31×10^{-5}**	**5.71×10^{-5}**	0.058	1
6	**0.051**	0.010	0.123	–

*Numbers in **boldface** indicate statistically significant differences (analysis of variance).

other zoonotic viruses, which presumably reflects long-term selection for resistance (*1*,*2*). This resistance is little studied and poorly understood, yet it might provide insight into improved treatments for arbovirus infections in humans. Our findings may also have implications for VEEV ecology, especially in the event of virus introduction into a nonenzootic region, as occurred during the 1971 Texas VEEV epizootic (*38*,*39*). During such a scenario, virus-induced deaths might deplete cotton rat populations, depending on the VEEV transmission levels and the length of the outbreak.

Viremia and Immunologic Responses

The differences in viremia profiles exhibited by the VEE-sympatric versus VEE-allopatric cotton rat populations could explain the different disease outcomes. Although peaks of viremia titers were similar for both subspecies, durations of viremia differed. The prolonged viremia observed in the Texas and Harlan animals may reflect a poor or delayed innate or adaptive immune response, which led to sustained viral replication and death. This could be an indirect effect of replication in lymphatic tissues leading to immunosuppression. Although peak viremia titers appeared to be age-dependent in the Florida population inoculated with the subtype IE VEEV strain, disease outcomes between age groups, which might reflect maturation of the immune system, did not differ significantly. Antibody titers in both populations of cotton rats after inoculation with either virus strain were relatively low.

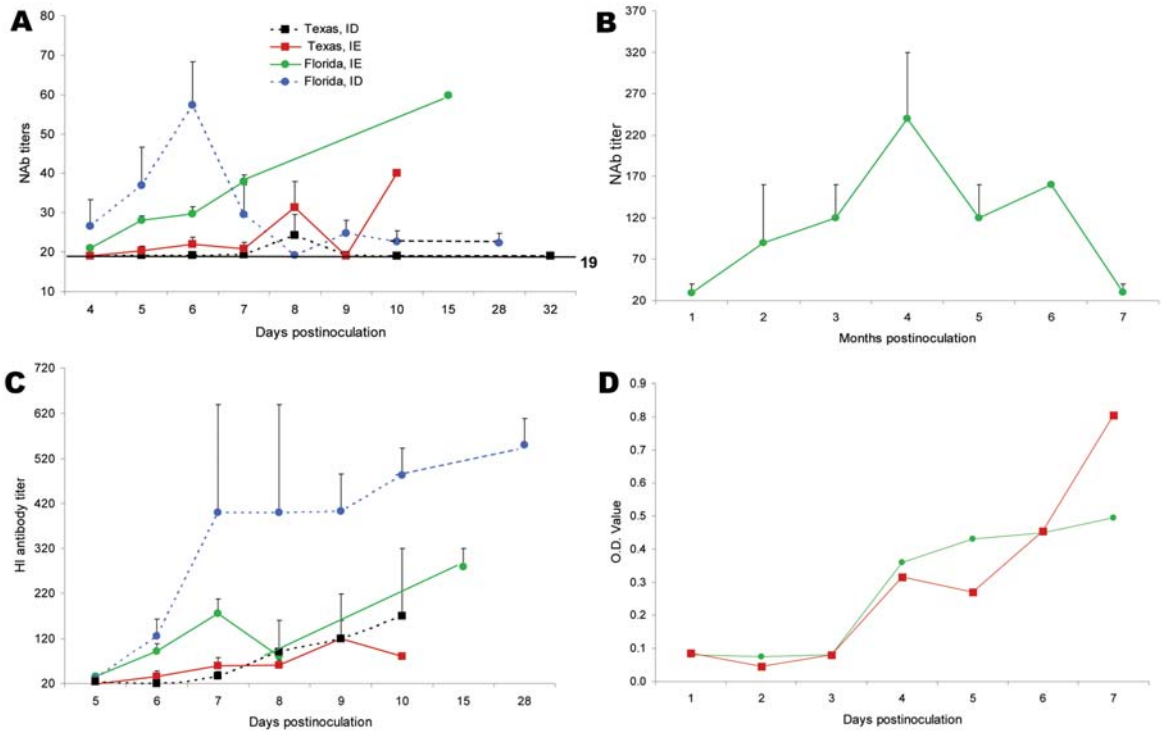

Figure 5. Antibody responses in cotton rats from Florida and Texas. A) Neutralizing antibody (NAb) titers in Florida group (n = 3–11) and Texas group (n = 1–17) inoculated with subtypes IE or ID Venezuelan equine encephalitis virus (VEEV). B) Long-term NAb titers in Florida rats infected with subtype IE VEEV (n = 2). C) Hemagglutination inhibition (HI) antibody titers for Florida (n = 2–10) and Texas (n = 1–16) rats inoculated with subtype IE VEEV. D) Immunoglobulin M antibody titers for Florida and Texas rats infected with subtype IE VEEV (n = 2). OD, optical density.

These findings contrast with results of a previously published study of EVEV infection of cotton rats from the same 2 geographic regions (*25*). In that study, both subspecies of rats survived infection, exhibited similar peak viremia titers, and had high antibody titers within 9 days postinoculation; it was suggested that the innate immune response was involved. EVEV, enzootic in South Florida, is less virulent in laboratory rodents than in most other viruses in the VEE complex, including the subtypes we used (*19*,*40*). Presumably due to this lack of virulence, Florida and Texas strains of cotton rats tested produced protective antibodies and survived EVEV infection (*25*).

Our data from the same 2 rat populations but inoculated with more virulent stains of VEEV present a different picture. Although the innate immune response may be involved as well, antibody detection correlated with the disappearance of viremia. The capability of Florida cotton rats to produce antibodies against VEEV early may allow them to better control replication and survive. Antibodies against VEEV persist for at least 6 months in laboratory-infected cotton rats (*25*). The average lifespan of cotton rats in nature is estimated to be ≈6–8 months (*41*).

To identify protective mechanisms in the Florida population, additional studies focusing more on the innate immune responses of enzootic and nonenzootic cotton rat populations are needed. This could be approached by crossbreeding the Texas and the Florida rats and studying the offspring. Elucidation of protective mechanisms may be useful for developing new strategies for treating human or equine infections.

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Source of Variant Creutzfeldt-Jakob Disease outside United Kingdom

Pascual Sanchez-Juan,*† Simon N. Cousens,‡ Robert G. Will,§ and Cornelia M. van Duijn*

We studied the occurrence of variant Creutzfeldt-Jakob disease (vCJD) outside the United Kingdom in relation to the incidence of indigenous bovine spongiform encephalopathy (BSE) and to the level of live bovines and bovine products imported from the UK during the 1980s and the first half of the 1990s. Our study provides evidence that a country's number of vCJD cases correlates with the number of live bovines it imported from the UK from 1980 to 1990 (Spearman rank correlation coefficient [r_s] 0.73, 95% confidence interval [CI] 0.42–0.89, p<0.001). Similar correlations were observed with the number of indigenous BSE cases (r_s 0.70, 95% CI 0.37–0.87, p = 0.001) and carcass meat imported from the UK from 1980 to 1996 (r_s 0.75, 95% CI 0.45–0.89; p<0.001) Bovine imports from the UK may have been an important source of human exposure to BSE and may have contributed to the global risk for disease.

In 1996 a new variant of Creutzfeldt-Jakob disease (vCJD) was described in the United Kingdom (*1*). By September 2006, 196 cases had been reported worldwide; most (162, 83%) occurred in the UK. Laboratory and epidemiologic studies provide strong circumstantial evidence for a causal link between vCJD and the bovine spongiform encephalopathy (BSE) epizootic in cattle (*2*,*3*) with the most likely route of primary human infection being through dietary exposure to highly infected bovine tissues (*3*).

In recent years, vCJD has been identified in a number of European countries with indigenous outbreaks of BSE, including 20 cases of vCJD in France, 4 in Ireland, 2 in the Netherlands, and single cases in Portugal, Italy, and Spain. A growing number of cases of vCJD have also been identified in countries outside Europe that have minimal incidence of BSE, including Japan, the United States, and Canada, and also in Saudi Arabia, a country in which BSE has not been identified (*4*). Several of the non-UK patients, notably 2 Irish, 1 Canadian, 2 American, and possibly the Japanese patient, may have been infected during periods of residence in the UK, but most cases (28 of 34) occurring outside the UK were in persons who had never visited the UK. Although vCJD cases have occurred in countries with very low incidence of BSE, no cases have been reported in countries with higher incidence of BSE, such as Switzerland (460 reported cases of BSE) and Germany (395 cases) (*5*). This fact raises questions as to the source of infection in the vCJD cases outside the UK. Were the patients exposed to indigenous cases of BSE or to infected bovine material imported from the UK? An analysis of infection risk in France suggests that the most likely source of vCJD in that country is imported infected material from the United Kingdom (*6*). In the Republic of Ireland, the transmission of BSE to humans was estimated to be equally likely from indigenous BSE or from UK imports (*7*). Given the apparently weak association between the occurrence of indigenous BSE and vCJD in some countries, we studied the occurrence of vCJD cases outside the UK in relation to the level of imported bovines and bovine products from the UK during the 1980s and the first half of the 1990s.

Methods

A European Union (EU) surveillance network, established in 1993, ensures prospective surveillance for CJD by using standard methods (*8*) in 18 European countries and the United States, Canada, Israel, and Australia. By August 24, 2006, 32 cases of vCJD had been identified in these countries (excluding the UK) (Table 1). Two more cases had been identified in countries outside the surveillance network, Japan and Saudi Arabia. Case-patients in Canada (n = 1), the United States (n = 2), Ireland (n = 2), and possibly Japan (n = 1) were considered as likely to have been infected during periods of residence in the UK.

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Table 1. Worldwide variant Creutzfeldt-Jakob disease cases as of August 2006

Country of residence at disease onset	N
United Kingdom	162
France	20
Republic of Ireland	2+2*
Italy	1
United States	2*
Canada	1*
Saudi Arabia	1
Japan	1*
The Netherlands	2
Portugal	1
Spain	1

*Most likely exposed to bovine spongiform encephalopathy in the UK.

Data on indigenous BSE cases detected by both passive and active surveillance were obtained from the World Organization for Animal Health webpage (www.oie.int/eng/info/en_esbmonde.htm) (*5*). The number of live cattle and the tonnage of carcass meat exported from the UK were derived from UK Custom and Excise Data (*9*).

We included in our analysis all countries covered by the EU surveillance network. Because exposure was expressed as a total amount rather than per capita, we based the analysis on number of vCJD cases rather than rates. We plotted the incidence of vCJD (number of cases) by country against the following factors: 1) number of cases of indigenous BSE, 2) number of live bovines imported from the United Kingdom from 1980 to 1990, and 3) tonnage of carcass meat imported from the United Kingdom from 1980 to 1996. For the last 2 plots, we used logarithmic scales to improve visualization of the data.

We have included carcass meat data until 1996, when all UK bovine imports were banned by the EU. However, for live bovine imports we limited our analysis to the period 1980–1990. In 1990 the EU restricted live bovine exports from the UK to animals <6 months of age and required that the importing country must ensure that any imported cattle from the UK were slaughtered at <6 months of age (*10*). For the correlation analyses, we included only the non-UK vCJD patients who are thought likely to have acquired infection outside the UK. A factor likely to be important for BSE exposure is the temporal distribution of UK exports. The number of BSE-infected cattle entering the human food supply is estimated to have peaked in the UK around 1989 (*7*), although with respect to exports, peak exposure may have been later, around 1992–1993. For example, 85% of Germany's imports of UK carcass meat between 1980 and 1990 were before 1988. In contrast, 70% of the livestock imports to the Netherlands were from 1987 through 1990. To account for this fact, we weighted the number of live bovines and the carcass meat tonnage imported each year by the size of the UK BSE epizootic that year (reported clinical cases) and normalized the data by the maximum number of BSE cases detected in a year during the epizootic. Nonparametric Spearman rank correlation coefficients (r_s) were calculated to evaluate whether evidence of a correlation between exposure and outcome existed.

Results

Figure 1 shows a scatter plot of the number of cases of indigenous BSE in non-UK countries and the number of non-UK vCJD cases per country. Although the confidence intervals (CIs) are wide, evidence of a correlation between these 2 variables (r_s 0.70, 95% CI 0.37–0.87, p = 0.001) exists in the countries belonging to the EU network (Table 2). When we included in our analysis Japan and Saudi Arabia, the only 2 countries outside the EU network in which vCJD cases have been detected, the correlation coefficient fell to 0.55 (95% CI 0.17–0.79, p = 0.008) (Table 2). Non-UK vCJD cases in EU network countries were also correlated with the number of live bovine imports from the UK (Figure 2; r_s 0.73, 95% CI 0.42–0.89, p<0.001) and the amount of carcass meat imported from the UK (Figure 3; r_s 0.75, 95% CI 0.45–0.89, p<0.001). Including Japan and Saudi Arabia produced similar results (live bovines r_s 0.65, 95% CI 0.31–0.84; carcass meat r_s 0.73, 95% CI 0.45–0.88) (Table 2).

We evaluated whether our findings were dependent on the data from France, which has the largest number of non-UK cases, by repeating the analyses excluding France. Evidence remained that all 3 exposures were correlated with vCJD incidence (Table 2). We also repeated the analysis including the 6 cases detected outside the UK but thought to have been acquired in the UK. The inclusion of these cases resulted in a reduction in all 3 correlation coefficients (Table 2).

Discussion

Live bovine and carcass meat imports from the United Kingdom during the 1980s and the first half of the 1990s correlate with the numbers of vCJD cases in countries

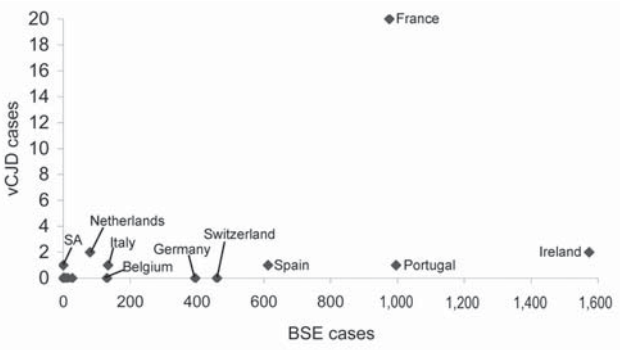

Figure 1. Scatter plot of the number of cases of indigenous bovine spongiform encephalopathy (BSE) in non–UK countries and the number of non-UK variant Creutzfeldt-Jakob disease (vCJD) cases per country. SA, Saudi Arabia.

Table 2. Results of nonparametric correlation analyses between number of variant Creutzfeldt-Jakob disease (vCJD) cases and the 3 studied exposure sources*

Countries and vCJD case-patients included in analysis	Exposure		
	Indigenous BSE cases (CI, p value)	Live bovines imported from UK, 1980–1990 (CI, p value)†	Carcass meat imported from UK, 1980–1996 (CI, p value)†
All EU network countries			
Patients likely to have been infected in UK excluded	r_s = 0.70 (CI 0.37–0.87, p = 0.001)	r_s = 0.73 (0.42–0.89, p<0.001)	r_s = 0.75 (0.45–0.89, p<0.001)
Patients likely to have been infected in UK included	r_s = 0.60 (0.21–0.82, p = 0.005)	r_s = 0.63 (0.26–0.84, p = 0.003)	r_s = 0.64 (0.27–0.84, p = 0.003)
All EU network countries plus Japan and Saudi Arabia			
Patients likely to have been infected in UK excluded	r_s = 0.55 (0.17–0.79, p = 0.008)	r_s = 0.65 (0.31–0.84, p = 0.001)	r_s = 0.73 (0.45–0.88, p<0.001)
Patients likely to have been infected in UK included	r_s = 0.51 (0.11–0.77, p = 0.02)	r_s = 0.52 (0.13–0.77, p = 0.01)	r_s = 0.57 (0.19–0.80, p = 0.006)
All countries except France			
Patients likely to have been infected in UK excluded	r_s = 0.48 (0.06–0.75, p = 0.03)	r_s = 0.60 (0.23–0.81, p = 0.005)	r_s = 0.68 (0.36–0.86, p = 0.001)
Patients likely to have been infected in UK included	r_s = 0.44 (0.01–0.73, p = 0.05)	r_s = 0.44 (0.01–0.73, p = 0.05)	r_s = 0.49 (0.08–0.76, p = 0.02)

*BSE, bovine spongiform encephalopathy; UK, United Kingdom; EU, European Union; CI, confidence interval; r_s Spearman rank correlation coefficient.
†Weighted by the temporal distribution of the export in relation to the size of the BSE epizootic in the UK.

outside the UK. This finding suggests that live bovine and/or carcass meat imports from the UK may have been an important source of exposure in at least some of the countries in which vCJD has been detected. These results are consistent with an analysis of data from France, which suggested that UK bovine imports were likely to have been a more important source of infection than indigenous BSE (6). Thus, a proportion of cases observed to date outside the UK may have been acquired through imports from the UK rather than by the patients' exposure to indigenous BSE. The inclusion in the analysis of the 6 non-UK case-patients thought to be infected in the UK reduced all 3 correlation coefficients, as one would expect if the supposition that they were infected in the UK is correct.

These findings come with several important caveats. First, they are based on small numbers of vCJD cases. Even a small number of additional non-UK cases in the future could alter the findings substantially. Furthermore, we have not performed multivariable analyses to determine which of the 3 exposures of interest were correlated with vCJD incidence because we were concerned that the small number of cases might lead to unreliable results. Second, the analyses of imports are based on UK Customs and Excise data, not all of which been validated by importing countries. Even if these data are reasonably accurate, the actual level of BSE infection entering the human food chain in importing countries cannot be estimated because many important unknown variables exist, such as the age distribution of imported live bovines, the age at slaughter of these animals, the culinary habits in each country, and the possibility that some of the UK imports may have been re-exported to other countries. Third, indigenous BSE-infected cattle entering the food supply will have gone undetected until the introduction throughout the EU of the active abattoir testing program for BSE in 2000/2001; even now, cattle in the early stages of infection are unlikely to be detected. Furthermore, the efficiency of BSE surveillance varies from country to country: underascertainment is likely in those countries with limited or no active testing programs.

It is noteworthy that none of the 162 UK patients with vCJD identified up to September 2006 were born after 1989, the year in which the specified bovine offal ban was introduced to minimize human exposure to BSE; however, 2 of the 34 non-UK vCJD case-patients were born after 1989. Measures equivalent to the UK ban on specified bovine offals were not introduced in many continental European countries until 2000.

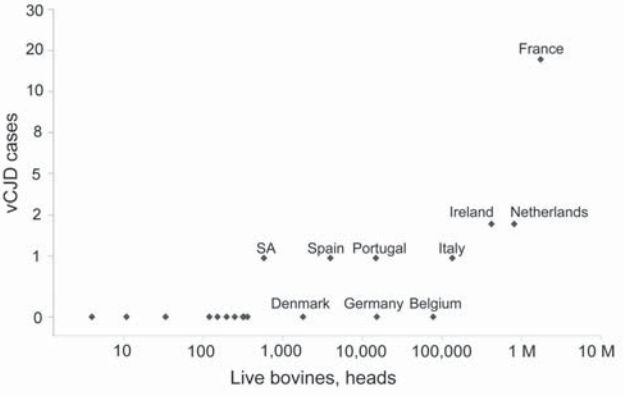

Figure 2. Scatter plot of live bovine imports (unweighted data) from the UK (1980–1990) and the number of non-UK variant Creutzfeldt-Jakob disease (vCJD) cases per country. Values are logarithmic. SA, Saudia Aradia; M, million.

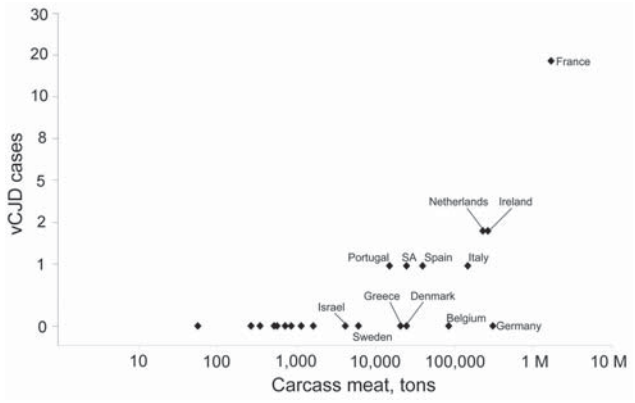

Figure 3. Scatter plot of the tonnage of carcass meat (unweighted data) imports from the United Kingdom (1980–1996) and the number of non-UK variant Creutzfeldt-Jakob disease (vCJD) cases per country. Values are logarithmic. SA, Saudia Aradia; M, million.

Despite these caveats, our results suggest that, globally, imports from the UK may have been an important source of infection and for some countries may even have been the main source. If this is so, our findings have several implications. Past UK exports may be the major determinant of the current incidence of vCJD outside the UK. The greatest volume of these exports was to France, the Netherlands, and Ireland. Thus, initially at least, we might expect the largest number of vCJD cases to occur in these countries. However, exposure to BSE through imports from the UK ceased in 1996, and exposure to indigenous BSE is likely to have continued at some level until the measures introduced in 2000. Thus, the proportion of vCJD cases due to exposure to indigenous BSE may increase with time.

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Infection with *Scedosporium apiospermum* and *S. prolificans*, Australia

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Scedosporium apiospermum and *S. prolificans* are fungi of increasing clinical importance, particularly in persons with underlying diseases. We reviewed the records of 59 patients in Australia from whom *Scedosporium* spp. were isolated from June 30, 1997, through December 31, 2003. *S. apiospermum* was isolated predominantly from the respiratory tracts of 28 of 31 patients with underlying lung diseases and resulted in 2 infections and 1 death. The annual number of *S. apiospermum* isolates remained constant. *S. prolificans* was isolated from 28 patients only after November 1999. Eight patients with acute myeloid leukemia or hematopoietic stem cell transplants had invasive infection; 4 had fungemia and 6 died from infection. *S. prolificans* caused locally invasive infection in 2 immunocompetent patients and was found in the respiratory tract of 18 patients with underlying respiratory disease but did not cause fungemia or deaths in these patients. *Scedosporium* spp. showed distinct clinical and epidemiologic features.

Scedosporium apiospermum and *S. prolificans* are saprophytic molds with a worldwide distribution. *S. apiospermum*, the anamorph of *Pseudallescheria boydii*, was described more than a century ago as a cause of Madura foot and subsequently mycetoma and otitis externa. Recently, it has been isolated from patients with chronic lung disease, particularly cystic fibrosis (*1*,*2*), where the spectrum of infection ranges from colonization to disseminated infection. *S. prolificans* was first described as a human pathogen in 1984 (*3*). *Scedosporium* spp. cause a broad spectrum of diseases, including soft tissue infections (*4*–*6*), septic arthritis (*7*), osteomyelitis (*8*,*9*), ophthalmic infections (*10*–*12*), sinusitis (*13*), pneumonia (*14*,*15*), meningitis and brain abscesses (*16*,*17*), endocarditis (*18*), and disseminated infection (*8*,*18*–*21*). Patients at risk include those immunocompromised because of advanced HIV infection (*6*,*18*,*20*), immunosuppressive therapy and neutropenia (*6*,*18*–*21*), or intravenous drug use (*6*,*20*).

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In the final months of 1999 during construction work, an increased number of *Scedosporium* spp. isolates and *S. prolificans* were noted at Alfred Hospital, a university hospital in Prahran, Victoria, Australia, that provides statewide trauma, burn, cystic fibrosis, heart and lung transplant, and HIV services. The records of all patients from whom *Scedosprium* spp. were isolated from June 30, 1997, through December 31, 2003, were reviewed to describe the epidemiology, clinical features, and outcomes of these infections.

Methods

Data Collection

Records of all patients with *Scedosporium* spp. cultured between June 30, 1997, and December 31, 2003, were reviewed retrospectively, and demographics, primary illness, antifungal therapy, and presence of other pathogens, and outcomes were recorded. Immunocompromised patients were defined as those with impairment of either or both natural and specific immunity to infection (*22*). Those with localized impaired host defenses caused by underlying diseases such as cystic fibrosis were classified as immunocompetent. Invasive infection was defined as a tissue biopsy specimen with hyphae plus culture of the organism (*23*). Disseminated infection was defined as positive blood cultures or infection at ≥2 noncontiguous sites within 7 days. Neutropenia was defined as an absolute neutrophil count <0.5 cells/mm^3. Descriptive statistics and odds ratios were calculated by using STATA release 8.0 (Stata Corporation, College Station, TX, USA.)

Laboratory Methods and Epidemiology

Scedosporium spp. were identified after culture onto horse blood agar and Sabaroud dextrose agar (SDA) and incubation at 30°C and 37°C. Colonies were rapid-growing, gray-white, and downy, with a gray-black reverse side. Species were differentiated by microscopic morphology (*24*). Susceptibility testing was performed according to Clinical and Laboratory Standards Institute methods (*25*) and synergy studies (2-dimensional 2-agent microdilution checkerboard method) (*26*). Synergy was a summation of the fractional inhibitory concentration ≤0.500.

For comparison of temporal patterns of mold isolates, the number of persons with *Scedosporium* spp. during the 3 years subsequent to this review (2004–2006) and *Aspergillus* spp. for 1999–2005 were extracted from laboratory databases. Rates of detection were expressed as per 1,000 separations and per 100,000 inpatient-patient days.

Environmental Sampling

From March 2001 through July 2002, environmental samples were collected from hospital public access areas within and adjacent to the construction site on 7 separate occasions. Additional sampling was conducted in ward corridors, nursing stations, patient rooms, and the patient car park. Air sampling was conducted with a portable high-volume air sampler (MAS 100; Merck, Darmstadt, Germany). The volume of air sampled at each site was 1,000 L/10 min (1 m^3). Settle plates were placed in ward corridors and patient rooms for 60 min of exposure. Dust was collected from horizontal surfaces with sterile swabs moistened with sterile saline. Dust and soil specimens were directly placed on standard SDA and selective SDA containing amphotericin B and incubated at 27°C.

Results

From June 1997 through December 2003, a total of 59 isolates of *Scedosporium* spp. were cultured from 56 patients. *S. apiospermum* was isolated from 31 patients, and *S. prolificans* was isolated from 28 patients. Both species were isolated from 3 patients at separate times (12 days, 13 months, and 27 months apart, respectively). Demographic information, coinfections, and outcomes of the patients are shown according to the species isolated (Table 1). During the period of this review, an average of 28 allogeneic stem cell, 35 lung, 27 heart, and 15 renal transplants were performed each year.

S. apiospermum

S. apiospermum was isolated from 32 specimens collected from 31 patients. Twenty-nine isolates were from the respiratory tract; sputum (n = 22), bronchoalveolar lavage (BAL) (n = 5), sinus (n = 1), and lung tissue (n = 1). The remaining 3 isolates were from brain tissue, a central venous catheter tip, and an ear swab, respectively. No blood cultures were positive for *S. apiospermum*. *S. apiospermum* was isolated concurrently with *Aspergillus* spp. from the respiratory tracts of 9 (29%) of the 31 patients.

Twenty-one (68%) of the 31 patients were immunocompromised. Thirteen had undergone solid organ transplantation (11 had lung transplants and 2 had heart transplants), 4 had malignancies (3 had metastatic cancer and 1 had chronic lymphocytic leukemia), 3 had advanced HIV infection, and 1 was receiving immunosuppressive therapy for rheumatoid arthritis. All 10 immunocompetent patients had chronic respiratory tract disease; cystic fibrosis (n = 5), bronchiectasis (n = 4) and chronic mastoiditis (n = 1).

Three patients were lost to follow-up after isolation of *S. apiospermum.* The remaining 28 patients were followed up for a total of 981 months. The mean duration of follow-up was 35 months (median 16 months, range 1–96 months). Two (6%) of the 31 patients had invasive infections. The first patient was a woman who received a lung transplant 5 years earlier and was previously colonized with *Aspergillus* spp. but not *S. apiospermum*. She had pulmonary and cerebral lesions and was treated with amphotericin B deoxycholate and itraconazole for presumed aspergillosis. *S. apiospermum* was isolated from postmortem lung and brain tissue. The second patient was a man who had advanced HIV infection with fungal sinusitis on biopsy. He was treated with voriconazole and surgery but died 3 months later from infection with cytomegalovirus. There were 6 deaths within 1 month of isolation of *S. apiospermum*. All deaths occurred in immunocompromised patients but only 1 was directly attributable to *S. apiospermum*. The other deaths resulted from the underlying condition.

Four patients with chronic lung disease were receiving itraconazole for treatment of *Aspergillus* spp. infection at the time *S. apiospermum* was isolated. Three of these patients died of respiratory failure 7, 9, and 16 months, respectively, after isolation of *S. apiospermum*, and 1 was alive when last seen 96 months after fungal isolation. Seven patients received antifungal therapy after isolation of *S. apiospermum* from respiratory tract samples (4 received voriconazole and 3 received itraconazole). These 7 patients had HIV infection and sinusitis (n = 2), had undergone lung transplantation (n = 3) or had bronchiectasis or cystic fibrosis (n = 2). Both patients with HIV infection had sinusitis and died within 7 months of complications of HIV. Of the other 5 patients, 4 who received azole therapy remained well without invasive infection at 32, 41, 48, and 88 months, respectively, of follow-up. The remaining treated patient died 15 months later; death was not attributed to fungal infection. The median duration of follow-up of those treated with azoles at the time of fungal isolation or subsequent to isolation was 16 months (range 3–96 months); *S. apiospermum* was not subsequently detected in these

Table 1. Characteristics and outcomes of patients infected with *Scedosporium* spp., Australia, 1997–2003

Characteristic	*S. apiospermum*	*S. prolificans*
No. patients	31	28
Patient demographics		
Mean age (range), y	40	42
Sex (M:F)	19:12	16:12
Immunocompromised (%)	21 (68)	14 (50)
Stem cell transplant	0	6
Hematologic malignancy	0	2
Lung and/or heart transplant	13	6
HIV infection, cancer, immunosuppression	8	0
Immunocompetent (%)	10 (32)	14 (50)
Cystic fibrosis	5	6
Airways disease*	4	3
Sinusitis	1	3
Other condition†	0	2
Specimen type		
Respiratory tract (%)	27 (68)	20 (71)
Tissue	3	6
Blood	0	4
Other‡	2	1
Additional microorganisms identified (%)	16 (42)	14 (50)
Molds§	9	10
Bacteria¶	9	8
Outcome at 1 mo		
Invasive infection (%)#	2 (6)	10 (36)
Died at 1 mo (%)**	6 (19)	5 (18)
Died of scedosporiosis (%)††	1 (3)	5 (18)
Attributable mortality rate of invasive disease (%)‡‡	1/2 (50)	5/10 (50)

*Bronchiectasis, asthma, and chronic obstructive pulmonary disease.
†Osteoarthritis and trauma.
‡Ear swab, central catheter tip, and synovial fluid.
§*Aspergillus* spp, *Rhizopus orrhyzae*, and *Paecilomyces* sp.
¶Bacteria not found in normal flora included *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Staphylococcus aureus*, *Nocardia* sp., and *Mycobacterium avium* complex.
#Odds ratio (OR) 6.64, 95% confidence interval (CI) 1.62–27.12, p = 0.001.
**OR 1.11, 95% CI 0.40–3.04, p = 0.84.
††OR 5.53, 95% CI 0.68–44.54, p = 0.06.
‡‡OR 1.0, 95% CI 0.21–4.56, p = 1.0.

patients. The median duration of follow-up for patients receiving no treatment after fungal isolation was 19 months (range 1–84 months); *S. apiospermum* was isolated from 4 of these patients 1, 18, 30, and 36 months, respectively, after initial fungal isolation.

S. prolificans

S. prolificans was isolated from 46 specimens obtained from 28 patients. Fourteen (50%) of the 28 patients were immunocompetent. Most (12/14, [86%]) specimens from immunocompetent patients were from the respiratory tract; cystic fibrosis (n = 6), chronic airway disease (n = 3), nasal discharge and sinus aspirates with chronic sinusitis (n = 3). Patients with cystic fibrosis or airway disease were not considered to have invasive disease and none received antifungal therapy. All 3 patients with chronic sinusitis were treated with surgery, and 2 received itraconazole. One isolate was from knee cartilage of a patient with hemophilia, osteoarthritis, and a knee replacement; the patient underwent surgery and received itraconazole. *S. prolificans* was also isolated from a skin-biopsy specimen from a patient with multiple-trauma and cellulitis who was treated only with surgery. There were no cases of disseminated infection or deaths resulting from *S. prolificans* in these immunocompetent patients.

S. prolificans was isolated from 14 immunocompromised patients. Immunodeficiency was caused by lung transplantation (n = 6), hematopoietic stem cell transplantation (HSCT) (n = 6), acute myeloid leukemia (AML), and myelodysplastic syndrome (MDS). *S. prolificans* was isolated from BAL of 6 lung transplant recipients. One patient was receiving itraconazole for *Aspergillus* spp. infection, 3 were receiving voriconazole, and 2 were receiving itraconazole and voriconazole with terbinafine. Invasive disease did not develop in any of the 4 lung transplant recipients who received antifungal treatment and none died. Two lung transplant recipients did not receive antifungal treatment. One of these patients was lost to follow-up and 1 survived >25 months without developing invasive infection, although *S. prolificans* was isolated again 1 year later.

S. prolificans was isolated from August 2000 through September 2002 from 6 patients undergoing allogeneic HSCT and from 2 patients with hematologic malignancy. Four of the 6 HSCT recipients had positive blood cultures, and 4 recipients had skin lesions (multiple, nodular). Findings for 8 patients in whom invasive disease developed are summarized in Table 2.

Patients 1, 2, and 6 had HSCT complicated by chronic extensive refractory graft versus host disease (GVHD). All 3 died of invasive infection despite treatment with itraconazole or voriconazole and terbinafine; 2 had positive blood cultures. Infections developed in patients 3, 4, and 5 during neutropenia after HSCT. Two of them had positive blood cultures, and both died. Patient 4 received no antifungal treatment, and patient 5 died despite replacement of prophylactic itraconazole with empiric amphotericin B. Patient 3, described elsewhere (*8*), survived after treatment with voriconazole, terbinafine, surgery, and neutrophil recovery. Patient 7 had MDS and preceding idiopathic CD4-cell lymphocytopenia. *S. prolificans* was isolated from sputum, but not BAL, and this patient was not treated. One month later, *S. prolificans* sinusitis was diagnosed. Despite surgery and treatment with itraconazole, followed by voriconazole and terbinafine, the patient died. Patient 8, a woman, was neutropenic after remission-induction chemotherapy for AML. A swab from a Hickman catheter exit site was positive for *S. prolificans*. Subsequently, chest wall cellulitis, deep soft tissue infection, and multiple skin nodules developed. She was treated with surgery, voriconazole, and terbinafine. Her neutropenia resolved and she recovered.

After isolation of *S. prolificans*, 5 of 28 patients were lost to follow-up. The remaining 23 were followed up for a total of 517 months. Mean length of follow-up was 22.5 months (median 9 months, range 1–68 months). Invasive infection occurred in 10 (36%) of 28 infected with *S. prolificans* compared with 2 (6%) of 31 infected with *S. apiospermum*. Deaths caused by scedosporiosis occurred in 5 (18%) of 28 infected with *S. prolificans* and 1 (3%) of 31 infected with *S. apiospermum*. All deaths occurred in immunocompromised patients; 6 (43%) of 14 infected with *S. prolificans* and 1 (5%) of 21 infected with *S. apiospermum* (odds ratio 6.0, 95% confidence interval 0.78–45.62, p = 0.05).

Drug-Susceptibility Testing

Drug-susceptibility testing was performed on 7 *S. prolificans* isolates. MICs (mg/L) were >16 for amphotericin B, >64 for fluconazole, >8 for itraconazole, >64 for 5-fluorocytosine, and >16 for ketoconazole, 2–8 for voriconazole, and 1–4 for terbinafine. Terbinafine and itraconazole were synergistic for 3 of 6 isolates tested, and terbinafine and voriconazole were synergistic for 5 of 7 isolates tested. Four *S. apiospermum* isolates tested

Table 2. Characteristics of 8 patients with invasive disease and *Scedospoeium prolificans* infections, Australia, 1997–2003*

Patient no.	Primary disease	Transplant type	Neutropenia within 30 d	GVHD	Date of first isolate	Days post transplant	Initial symptom	Site of isolates	Outcome following diagnosis
1	ALL	Allogeneic HSCT	Yes	Chronic extensive	Aug 2000	>400	Knee effusion	Blood, synovium, cartilage, prostate	Died d 21
2	AML	Allogeneic HSCT	No	Chronic extensive	Apr 2001	>500	Pulmonary infiltrate	Blood, sputum, BAL, lung	Died d 5
3	MM	Allogeneic HSCT	Yes	No	Nov 2000	28	Ethmoid sinus infiltrate	Ethmoid sinus, vertebral disc, mycotic aneurysm	Alive d 500
4	NHL	Allogeneic HSCT	Yes	No	Dec 2000	10	Neutropenic sepsis	Blood	Died before diagnosis
5	AML	Allogeneic HSCT	Yes	No	Mar 2003	10	Neutropenic sepsis	Blood, sputum, BAL, lung, skin	Died d 1
6	MM	Allogeneic HSCT	Yes	Chronic extensive	Nov 2002	120	Pneumonia	Sputum	Died d 1
7	MDS	NA	No	NA	Nov 2003	NA	Maxillary sinus infiltrate	Sputum, maxillary sinus, pericardium, myocardium, kidney, skin, lung	Died d 14
8	AML	NA	Yes	NA	May 2002	NA	Catheter-related sepsis	Chest wall, Hickman catheter	Alive d 500

*GVHD, graft versus host disease; ALL, acute lymphoblastic leukemia; HSCT, hematopoietic stem cell transplantation; AML, acute myeloid leukemia; BAL, bronchoalveolar lavage; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; MDS, myelodysplastic syndrome; NA, not applicable because patients did not undergo stem cell transplantation.

had MICs of 0.5–1 mg/L for itraconazole and 0.5 mg/L for voriconazole.

Epidemiology of *Scedosporium* spp. and *Aspergillus* spp.

Throughout the study, *S. apiospermum* was isolated at a constant rate (Figure) of 3–6 isolates per year with no seasonal clustering. In contrast, *S. prolificans* was first detected in December 1999, and thereafter ≈8 isolates were identified annually. Detection of *S. prolificans* continued in 2004 with 8 isolates but decreased to 6 in 2005 and 4 in 2006. Two clusters of infection occurred in autumn 2001 and 2003 during periods of hospital building that required deep excavation. Air, dust, and soil sampling did not detect an environmental fungal source. *Aspergillus* spp. were detected during 1999–2005 (mean 191 persons per year, range 153–230). Detection rates for *Scedosporium* spp. and *Aspergillus* spp. for 2003–2005 were 0.1–0.23/1,000 separations and 2–3/100,000 inpatient-patient days and 2.4–3.0/1,000 separations and 53–75/100,000 inpatient-patient days, respectively.

Discussion

This study describes the epidemiology of *Scedosporium* infection in a cohort of contemporaneous patients at a university hospital. The single-center approach, with cases identified by laboratory detection, allows capture of the full spectrum of infection from colonization to invasive infection. *Scedosporium* spp. were detected in a broad range of patients and clinical settings. However, there were distinct differences in epidemiology, clinical manifestations, antifungal susceptibility patterns, and outcomes between *S. prolificans* and *S. apiospermum*.

Previous reviews have focused on invasive cases reported (*10*,*27*,*28*), but this approach is limited by both selection and publication bias (*29*) and does not describe the natural history of infection or the prevalence of these infections. Our series enables the annual incidence of cases, ratio of colonized to infected patients, and natural history of colonization to be determined for each species. We showed that invasive infections accounted for only 6% of *S. apiospermum* isolates and for 46% of *S. prolificans* isolates, and that isolation of *Aspergillus* spp. was 20–30 times more frequent than that of *Scedosporium* spp.

S. apiospermum and S. prolificans are colonizers of abnormal airways caused by bronchiectasis, cystic fibrosis, chronic obstructive pulmonary disease, or lung transplantation (*1*,*2*,*6*,*18*,*30*,*31*). *S. apiospermum* was an airway colonizer in ≤10% of patients with cystic fibrosis in France and Australia (*1*,*2*), and similar proportions of lung transplant recipients were colonized with 1 or both species at 1 center in Australia (*31*). However, there are few reports that *S. prolificans* colonized patients with lung disease (*6*,*32*). In the present study, *S. prolificans* was identified as an airway colonizer only after December 1999. The onset of invasive infections in HSCT and neutropenic patients occurred in August 2000. *S. prolificans* was first detected at the M.D. Anderson Cancer Center in Houston, Texas, after 2001 (*19*). Emergence of *S. prolificans* may be related to an environmental source that was not identified or selection pressure caused by changes in antifungal prophylaxis practices (*33*). This finding did not appear to be the explanation in our patients because fluconazole remains standard prophylaxis for neutropenia, and itraconazole was used in only 1 of 8 patients. Other possible explanations include use of more aggressive chemotherapy regimens in patients with acute leukemia and the advent of nonmyeloablative allografts, which change characteristics of patients selected for transplants. The reason for persistence of this organism over several years after its initial appearance is also unclear but has also been observed by others (*19*).

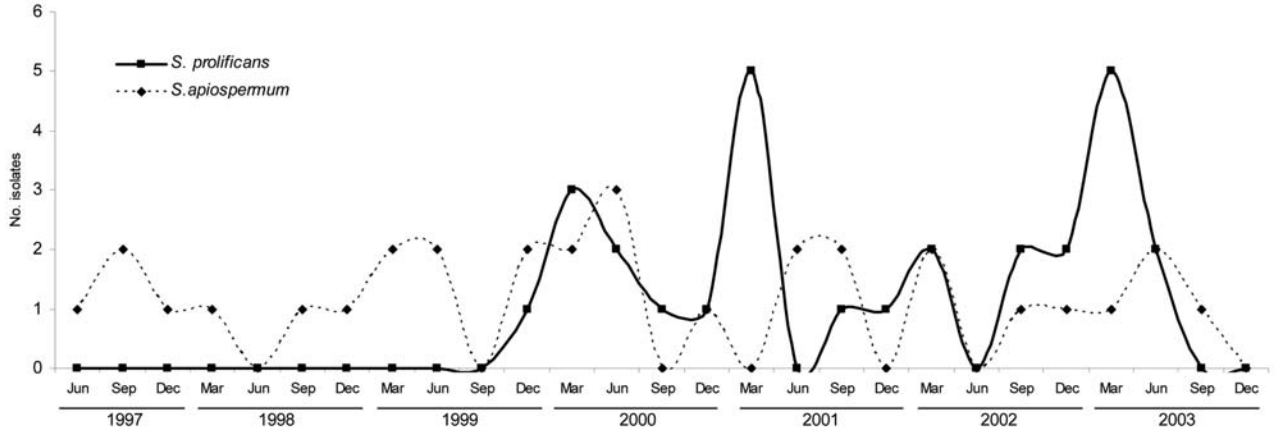

Figure. Epidemiologic curve of isolation of *Scedosporium* spp. isolation, Australia, June 1997–December 2003. *S. prolificans* was first identified in December 1999 and had 2 peaks that coincided with construction work. *S. apiospermum* was isolated at a constant rate of 1–2 times per 3-month period.

In patients with respiratory tract disease, both *Scedosporium* spp. were isolated in comparable numbers. However, the only invasive infection in this diverse group was disseminated *S. apiospermum* 5 years after lung transplantation. The outcome of 19 patients who had undergone lung or heart lung transplantation and were colonized with this fungus was comparable to the 17 patients in the immunocompetent group with airways disease caused by cystic fibrosis, bronchiectasis, or chronic obstructive pulmonary disease. As in other reports, other opportunistic pathogens, especially *Aspergillus* spp. (present in one third), were commonly cultured simultaneously (*1*,*18*,*30*,*31*). In lung transplant recipients, *Scedosporium* spp. in BAL was associated with advanced bronchiolitis obliterans and airway stenosis (*31*), which emphasizes the difficulty of interpreting the role of *Scedosporium* spp. in this group. Whether colonization follows airway damage, immunosuppression, or antifungal therapy to treat *Aspergillus* spp. colonization or is itself an ongoing cause of airway damage such as bronchiolitis obliterans is unknown. In our study group, most patients with respiratory tract colonization had not received antifungal therapy. Although antifungal treatment was used in ≈50% of patients with *S. apiospermum* airway colonization, there appeared to be no survival advantage in patients treated; our study was not a randomized comparison. With *S. prolificans* respiratory tract colonization, too few patients were treated with antifungal drugs for valid conclusions to be made. There was no reduction in survival in 5 untreated patients with isolates of either *S. apiospermum* or *S. prolificans*. The role of *Scedosporium* spp. respiratory tract colonization requires further evaluation.

Allogeneic HSCT and AML/MDS were the only settings in which *S. prolificans* was more common than *S. apiospermum.* There were no *S. apiospermum* infections in these patients, although in at 1 US cancer center, *S. apiospermum* infections were more common (*19*). This may reflect different geographic distributions of *Scedosporium* spp. In our study, *S. prolificans* caused illness with high mortality rates in HSCT and leukemia patients. In HSCT recipients, *S. prolificans* infections occurred equally in those with neutropenia (early after transplant) and GVHD (later after transplant). Thus, host factors unique to AML and HSCT, such as neutropenia, GVHD, associated macrophage dysfunction, and environmental exposure, may play a role in the propensity for invasive infection with *S. prolificans* in this group.

As in previous reports (*10*,*32*), neutropenic patients had sepsis, positive blood cultures, and overwhelming infection (often associated with disseminated rash). In nonneutropenic patients, infection was more likely to be localized initially in lungs, joints, or sinuses. The ability of *S. prolificans* to grow in blood cultures in AML and HSCT patients is well recognized (*10*,*34*), and this diagnosis, or infection with *Fusarium* spp., should be considered when a mold is cultured from blood. In our study, *S. prolificans* was the only species to grow in blood culture, although others have reported *S. apiospermum*, albeit, less frequently (*10*,*18*,*19*). Although colonization with *S. prolificans* without progression to disease was observed in patients with respiratory disease and after lung transplantation, this was not observed in 3 patients with AML/MDS or HSCT. A nonsterile site swab or sputum culture yielding *S. prolificans* was soon followed by a diagnosis of invasive infection, which indicated that in these patients a culture result, even from a nonsterile site, should be viewed seriously.

The clinical findings of *S. prolificans* in our patients suggest that this fungus is more invasive than *S. apiospermum*, as has been found in mice (*35*). In addition to disseminated infection in AML patients and HSCT recipients, *S. prolificans* also caused locally invasive disease in patients who were not immunocompromised, such as those with posttrauma cellulitis, septic arthritis in a damaged joint, and sinus disease. One proposed mechanism for virulence of *S. prolificans* is melanin in the cell wall (*27*).

Antifungal therapy is problematic with *S. prolificans* with high MICs for amphotericin B, echinocandins, and azoles. This finding has stimulated interest in combinations of voriconazole and terbinafine (*26*), voriconazole and echinocandin (*36*), or polyene and echinocandin (*36*). However, these infections are rare and experience is limited to case reports (*8*,*9*,*36*). In vitro treatment with interferon-γ and granulocyte-macrophage–colony-stimulating factor has improved neutrophil function against *S. prolificans* hyphae (*37*). Surgery appears to be an important factor in survival in our cases, as well as other series (*10*). Although surgery and antifungal therapy were successful in 2 neutropenic patients with invasive *S. prolificans* infections, these patients also had concomitant neutrophil recovery that could explain their survival.

Much needs to be learned about the epidemiology, transmission, pathogenesis, and environmental niche of *Scedosporium* spp., especially *S. prolificans*. A source for emergence of *S. prolificans* at our hospital was investigated, but none was identified. Our inability to detect the source of emergent *S. prolificans* was puzzling, although exposure and colonization may have occurred by the time the first case was seen. Cultures may have been obtained too late to identify environmental contamination, although changes in immunosuppression in the population at risk may also have contributed. *S. prolificans* appeared first as a colonizer of abnormal airways 8 months before the first invasive infection in an HSCT recipient. Shifts in the epidemiology of colonizing organisms in this patient group during hospital construction may be worthy of closer attention and surveillance. Molecular typing methods such as PCR (*38*) and inter-simple sequence repeat PCR fingerprinting (*39*)

have distinguished genotypes from outbreaks and different geographic regions. Recently, *S. apiospermum* complex has been shown to include several individual species indistinguishable morphologically (*40*). These methods will be used for further investigation of our isolates as part of a larger Australian surveillance study.

In conclusion, *S. prolificans* and *S. apiospermum* are pathogenic fungi that demonstrate distinct clinical features dependent on the immune function of the host and the type of species isolated. They are usually found in patients with underlying disease, although occasionally after trauma. *S. prolificans* emerged as a major pathogen in allogeneic HSCT recipients and as a colonizer of patients with underlying lung disease. Madura foot is now rare and was not observed in this series. The high attributable mortality rate of invasive infection with both *Scedosporium* spp., limitations of antifungal therapy, necessity for aggressive and deforming surgery to treat infections with *S. prolificans*, and uncertainty over the role of airway colonization emphasize the need to better understand the epidemiology and pathogenesis of this infection.

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Dr Cooley is a medical microbiologist and director of Infectious Diseases at Royal Hobart Hospital. Her research interests include diagnosis and management of infections in the immunocompromised host.

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Genetic Diversity of *Bartonella henselae* in Human Infection Detected with Multispacer Typing

Wenjun Li,* Didier Raoult,* and Pierre-Edouard Fournier*

We applied multispacer typing (MST) by incorporating 9 variable intergenic spacers to *Bartonella henselae* DNA detected in lymph node biopsy specimens from 70 patients with cat-scratch disease (CSD), in cardiac valve specimens from 2 patients with endocarditis, and in 3 human isolates from patients with bacillary angiomatosis, CSD, and endocarditis. Sixteen MST genotypes were found, 5 previously identified in cats and 11 new. Of the studied DNA, 78.7% belonged to 2 genotypes, which were phylogenetically organized into 4 lineages. Human strains were mostly grouped within 2 lineages, previously identified as Marseille and Houston-1. Our results suggest a greater genetic diversity in human-infecting *B. henselae* than what has previously been evaluated by using other genotyping methods. However, the diversity is not significantly different from that of cat strains. MST is thus a suitable genotyping tool for evaluating the genetic heterogeneity of *B. henselae* among isolates obtained from human patients.

Bartonella henselae, first identified in 1990 and characterized as a new species in 1992, is a gram-negative, fastidious bacterium associated with cats. *B. henselae* infection in cats is usually asymptomatic, but infected cats may remain bacteremic for long periods, thus playing a major role as a reservoir for the bacterium (*1*,*2*). Transmission of *B. henselae* among cats may be mediated by the cat flea, *Ctenocephalides felis* (*3*), and to humans by cat scratches or bites (*4*). *B. henselae* infection in humans exhibits a variety of clinical syndromes including the most common, cat-scratch disease (CSD) (*5*), endocarditis (*6*), bacillary angiomatosis (*7*) and peliosis hepatis in immunocompromised patients (*8*), and other less frequent manifestations (*9*). *B. henselae* has also been detected in various domestic or wild animals, including dogs, lions, panthers, and cheetahs (*10*). More recently, *B. henselae* was detected in the porpoise, a marine mammal (*11*).

Because *B. henselae* has a complex and expanding host or reservoir system and has been associated with a rapidly increasing spectrum of clinical syndromes (*12*), epidemiologic survey and exploration of population structure of this organism are critical. The following techniques have been used for this purpose: pulsed-field gel electrophoresis (PFGE); restriction fragment length polymorphism; enterobacterial repetitive intergenic consensus (ERIC)–PCR; repetitive extragenic palindromic (REP)–PCR; infrequent restriction site (IRS)–PCR (*10*,*13*–*18*); DNA sequencing–based approaches represented by sequences of 16S rDNA (*19*,*20*), *ftsZ* (*21*), *gltA* (*22*), 35-kDa protein–encoding (*23*), *groEL*, and *pap*31 genes (*24*); and the 16S-23S intergenic spacer (*25*). These investigations gradually offered insight into the population structure of *B. henselae* and allowed several genetic groups to be identified. Initially, *B. henselae* isolates were classified within 2 16S rRNA–based genotypes, I and II, and 2 serotypes, Marseille and Houston-1 (*20*,*23*,*26*). Studies that used *gltA*, *groEL*, and *pap*31 gene sequence analysis, arbitrarily primed–PCR, ERIC-PCR, IRS-PCR, PFGE, or multilocus sequence typing (MLST) were congruent with serotypes, but not with genotype I and II classification (*13*,*22*–*24*,*27*–*29*). Altogether, *B. henselae* isolates were found to be distributed within 3 distinct lineages (Marseille, Houston-1, and Berlin-2), and the 16S rRNA gene was not a sensitive marker of the clonal divisions of *B. henselae*. This suggested that a 16S rRNA recombination occurred in this species that may be facilitated by the coexistence of several strains in the blood of cats (*27*).

Several studies have suggested that human-associated isolates were less genetically heterogeneous than cat isolates (*22*,*27*,*30*), and a small group of cat isolates

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were hypothesized to be the source of human infection (*22*,*27*). However, the limited discriminatory power of the available genotyping methods, with a maximum of only 7 genotypes identified (*27*), and the small number of human isolates studied prevented the population structure and the genetic relationship between cat and human isolates of *B. henselae* to be reliably investigated. Recently, we applied multispacer typing (MST), using 9 highly variable intergenic spacers, to 126 *B. henselae* cat isolates and identified 39 MST genotypes, which made it the highest resolution typing system (available from http://ifr48.timone.univ-mrs.fr/mst_bhenselae/mst) (*31*). In this study, we applied MST, based on sequences from the 9 previously described variable intergenic spacers (*31*), to 75 human-infecting *B. henselae* isolates to evaluate their genetic diversity and compare it to that of cat isolates.

Materials and Methods

Study Design

We included in the study, when available, lymph node biopsy specimens and cardiac valve specimens from patients with a clinical diagnosis of CSD or endocarditis who had been referred to our laboratory from 2004 to 2006. All specimens were then frozen at –80°C for further culture and molecular detection of *Bartonella* species. *B. henselae* was detected by using PCR targeting the 16S-23S rRNA spacer (ITS) and *pap*31 gene (*24*,*25*). In addition, 3 human *B. henselae* isolates—type strain Houston-1, isolated from the blood of a patient with bacillary angiomatosis in USA; type strain Marseille, isolated from the lymph node biopsy specimen of a patient with CSD in France; and strain URBH-TOIE49, isolated from the valve biopsy specimen of a patient with endocarditis in France (*26*)—were incorporated in this study. All *B. henselae* isolates and PCR amplicons were tested by MST by using 9 variable intergenic spacers named S1-S9, from the most to the least variable among cat isolates (*31*). When available, the 16S rRNA genotypes of studied strains were indicated (online Appendix Figure, available from www.cdc.gov/EID/content/13/8/1178-appG.htm).

Culture Conditions of *B. henselae* Human Isolates

B. henselae isolates were cultivated on blood agar (bioMérieux, Marcy l'Etoile, France) at 37°C in 5% CO_2 (Genbag CO_2 System, bioMérieux). After a 5-day incubation period, *Bartonella* cells were collected for DNA extraction.

DNA Preparation

Total genomic DNA was extracted from the 3 studied *B. henselae* isolates by using the Chelex procedure as previously described (*32*). For patients' specimens, we used the QIAamp Tissue kit (QIAGEN, Hilden, Germany) to extract the total genomic DNA, as described by the manufacturer.

PCR Amplification and Sequencing

ITS and *pap*31 gene PCR amplifications were performed by using the previously described conditions and the 16SF-23S1 (*25*) and PAPn1-PAPn2 (*24*) primer pairs, respectively. For amplification and sequencing of the 9 intergenic spacers, we used the same primers as in our previous study (*31*), except for primers amplifying spacers S1 and S8, which caused unspecific amplifications from DNA extracted from human specimens. One new set of primers was designed to amplify each of these 2 spacers, as indicated in the online Appendix Table (available from www.cdc.gov/EID/content/13/8/1178-appT.htm).

These new primers also amplified the whole intergenic spacers and thus did not affect genotype comparison between human and cat strains. All primers were obtained from Eurogentec (Seraing, Belgium). PCRs were performed in a PTC-200 automated thermal cycler (MJ Research, Waltham, MA, USA). One nanomolar concentration of each DNA preparation was amplified in a 25-μL reaction mixture containing 50 pmol/L of each primer; 200 μmol/L (each) dATP, dCTP, dGTP, and dTTP (Invitrogen, Gaithersburg, MD, USA); 1.5 U of HotstarTaq DNA polymerase (QIAGEN); 2.5 μL 10× PCR buffer; and 1 μL 25 mmol/L $MgCl_2$. The following conditions were used for amplification: an initial 15-min step at 95°C was followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C. Final amplification was completed by holding the reaction mixture for 5 min at 72°C to allow complete extension of the PCR products. PCR products were purified by using the MultiScreen PCR filter plate (Millipore, Saint-Quentin-en-Yvelines, France) as recommended by the manufacturer. Amplicons were sequenced in both directions by using the BigDye 1.1 chemistry (Applied Biosystems, Foster City, CA, USA) on an ABI 3130XL automated sequencer (Applied Biosystems) as described by the manufacturer. To avoid contamination, no positive control was used. Sterile water was used as a negative control in each PCR. Sequences from each DNA sample were checked twice in both directions to ensure the reliability of the MST method.

Sequence Analysis

Nucleotide sequences were edited by using the Autoassembler package (PerkinElmer, Waltham, MA, USA). For each intergenic spacer, a genotype was defined as a sequence exhibiting unique mutations, which were checked by sequencing the corresponding spacers 3 times. MST genotypes were defined as unique combinations of the 9 spacer genotypes.

Phylogenetic Analysis

For the phylogenetic analysis, we concatenated sequences from the 9 studied spacers. We included in the analysis the 39 MST genotypes previously obtained from the MST analysis of cat isolates (*31*) and those, when different, obtained in the present study. Multiple alignment of sequences was carried out by using CLUSTALW software (*33*). Phylogenetic relationships were obtained by using the neighbor-joining and maximum parsimony methods within the MEGA 2.1 software (*34*), and the maximum likelihood method within the BioEdit software (available from www.mbio.ncsu.edu/bioedit/bioedit.html).

Statistical Analysis

The genotypic diversities of human and cat strains of *B. henselae* were compared by using the Fisher exact test within the Epi Info 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA). A difference was considered statistically significant when $p<0.01$.

Results

Genotypic Distribution of 75 *B. henselae* Strains Detected in Humans

From January 2004 to May 31, 2006, *B. henselae* was detected in 70 lymph node biopsy specimens from 70 patients with CSD and 2 cardiac valve specimens from 2 patients with endocarditis by using PCRs targeting the ITS and *pap*31 gene. Among the 72 *B. henselae*–positive samples, 71 were obtained from patients living in France, and the remaining specimen was from a cardiac valve biopsy from a patient living in Guadeloupe Island in the West Indies. When applying MST to the 72 *B. henselae* amplicons and the 3 studied isolates, we identified the following number of types for the S1–S9 spacers: 3, 5, 3, 4, 3, 2, 3, 2, and 2 types, respectively (Table). For each of spacers S1, S2, S4, and S6, a new genotype, i.e., 10, 8, 6, and 5, respectively, was identified. For each spacer, differences among genotypes mainly consisted of nucleotide substitutions. Ranges of nucleotide similarity rates among tested strains were 98.5%–100% for spacer 1, 97.4%–99.7% for spacer 2, 98.3%–99.7% for spacer 3, 99.1%–99.8% for spacer 4, 98.9%–100% for spacer 5, 97.8%–99.7% for spacer 6, 98.8%–99.6% for spacer 7, 98.3%–99.4% for spacer 8, and 97.6%–99.4% for spacer 9. Combining genotypes obtained from the 9 spacers allowed the 75 studied human *B. henselae* strains to be classified into 16 MST genotypes. Of these, 5 MST genotypes, including 64 strains, were previously known among cat isolates, i.e., types 5, 16, 22, 26, and 31 (online Appendix Figure). The remaining 11 MST genotypes, numbered types 40–50 including 1 human strain each, were new.

Sequences from the 4 new genotypes of spacers S1, S2, S4, and S6 and the 11 new MST genotypes were deposited in the GenBank database under accession nos. EF017703 (tRNA-*Ala*/GCA-tRNA-*Ile*/AUC spacer, type 10), EF017704 (BH2865724-*dut* spacer, type 8), EF017705 (*pssA*-Oxidoreductase spacer, type 6), and EF017706 (*alr-gcvP* spacer, type 5). These sequences were then added to the MST Rick database.

Phylogenetic Analysis

Phylogenetic trees obtained by using alignment of the 9 concatenated spacer sequences and the neighbor-joining, maximum parsimony, and maximum likelihood methods showed similar organizations. The 50 MST genotypes (in-

Table. Human-infecting *Bartonella henselae* included in this study and corresponding genotypes*

No. patients by clinical condition			Genotypes									
CSD	Endocarditis	Bacillary angiomatosis	S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
21	1		5	1	1	1	2	2	2	1	1	5
1†			5	2	1	1	2	2	2	1	1	16
37			3	5	4	5	2	2	2	1	3	22
3			5	1	1	5	2	2	2	1	1	26
1			5	1	1	1	2	2	4	2	1	31
		1‡	3	5	5	4	1	5	1	1	3	40
	1§		5	6	1	1	2	2	2	1	1	41
1			5	1	1	1	5	2	4	2	1	42
1			5	8	1	1	2	2	4	2	1	43
1			5	1	1	1	2	2	4	1	1	44
1			5	5	1	5	2	2	2	1	1	45
1			5	1	4	1	2	2	2	1	1	46
1			3	1	4	1	2	2	2	1	3	47
1			3	5	4	6	2	2	2	1	3	48
1			10	1	1	1	2	2	2	1	1	49
	1		5	2	5	4	1	2	1	1	1	50

*CSD, cat-scratch disease; MST, multispacer typing.
†Marseille strain.
‡Houston-1 strain.
§URBHTOIE 49 strain.

cluding the 39 MST genotypes previously identified among cat isolates [*31*] and the 11 new MST genotypes identified in the present study) were grouped into 4 clusters (online Appendix Figure). Two clusters were associated with previously described Houston-1 and Marseille serotypes. Cluster 1 was composed of 19 MST genotypes and contained 22 American and all 19 Asian cat isolates, and only 1 amplicon detected in the cardiac valve of the patient with endocarditis from Guadeloupe Island. Cluster 2 (Houston-1) comprised 6 MST genotypes and contained 17 European and 5 American cat isolates as well as 39 amplicons, including 38 from patients with CSD and type strain Houston-1. Cluster 3 (Marseille) included 21 MST genotypes represented by 8 European and 38 American cat isolates and 35 amplicons, including 33 from patients with CSD, 2 from patients with endocarditis. and type strain Marseille. Cluster 4 contained 13 European and 4 American cat isolates but no human amplicon (online Appendix Figure).

Two genotypes (5, and 22,) were mainly found in the Houston-1 and Marseille clusters, respectively. Genotype 22 contained 37 human strains or amplicons and 16 European cat isolates; genotype 5 included 22 human amplicons, 7 European and 23 American cat isolates. The 3 human strains from patients with endocarditis were classified within 3 different MST genotypes, i.e., genotypes 22, 41, and 50. Type strain Houston-1, obtained from a patient with bacillary angiomatosis, exhibited the unique genotype 50, whereas type strain Marseille shared genotype 16 with an American cat isolate (online Appendix Figure).

The genotypic diversity of human strains was not statistically different from that of cat isolates (16/75 vs. 39/129, $p = 0.3$), even when restricted to French human strains or cat isolates (14/73 vs. 6/29, $p = 0.9$). However, when we compared the distribution of human strains among clusters, we found that the Houston-1 and Marseille clusters contained significantly more human strains than did cluster 1 ($p<0.01$) and also that the Houston-1 cluster contained a significantly higher proportion of human strains than the Marseille cluster ($p<0.01$).

In addition to type strains Houston-1 and Marseille, the 16S rRNA genotype was known for 67 cat strains. Phylogenetic cluster 1 contained 18 type I strains; cluster Houston-1 contained 21 type I strains and 1 type II strain; and clusters Marseille and 4 contained 20 and 9 type II strains, respectively.

Discussion

We report the successful adaptation of MST to *B. henselae* detected in human samples. Isolating *B. henselae* from CSD patients is extremely difficult (*35*). In this context, a reliable and reproducible molecular typing method, using PCR coupled to sequencing, to study the genetic diversity of *B. henselae* detected in human specimens directly, is a valuable option. Recently, MLST that used 9 housekeeping genes classified 20 cat and 17 human isolates into 7 types, with most of the 17 human isolates belonging to 1 specific genotype. That study suggested that human isolates were more homogenous than cat isolates. However, the study was limited by the small number of human isolates studied and the limited discriminatory power of MLST, which hindered in-depth exploration of the genotypic diversity of *B. henselae* (*27*). In our present study with MST, using 9 highly variable ITS exhibiting a high resolution for subtyping *B. henselae* (*31*), we investigated the genetic diversity of *B. henselae* detected in humans.

When MST was previously applied to *B. henselae* cat isolates, we found 39 distinct genotypes, 4 of which (5, 2, 22, and 35) were predominant (*31*). We identified 16 MST genotypes among the 75 *B. henselae* human strains. Of these, 59 strains (78.7%) also belonged to genotypes 5 and 22. We found no statistical difference in genotypic diversity between the 75 human strains (16 MST genotypes) and the 126 previously studied cat isolates (39 genotypes, $p = 0.14$) (*31*).

The addition of 11 new MST genotypes to the 39 previously identified did not modify the phylogenetic distribution of 4 main clusters (lineages) described among cat isolates (*31*). However, the human strains had a specific phylogenetic distribution. Clusters 1 and 4 contained significantly fewer human strains than did clusters Houston-1 and Marseille ($p<0.01$). For cluster 1, this difference may be explained by the fact that this cluster contained only cat isolates from the United States and Asia, whereas we studied mostly human strains from France. The only human strain classified within cluster 1 was detected in 1 patient from the West Indies, but we acknowledge that this single patient, although exhibiting a specific genotype, does not allow us to draw any conclusions about the distribution of *B. henselae* genotypes in this area. Therefore, estimating the MST genotypes of human strains from the West Indies, United States, and Asia and comparing them to MST genotypes classified within cluster 1 might be useful. Regarding the distribution of strains within cluster 4 (4 cat strains from the United States, 10 cat strains from France, and 3 cat strains from Germany, but no human strain), we speculate that these strains may be less pathogenic for humans or that a sampling bias occurred. Thus, additional human and cat *B. henselae* isolates or amplicons from more countries will be needed to investigate the geographic correlation between human and cat isolates.

Previously, on the basis of the polymorphisms of the *pap31* gene, among 107 *B. henselae* human strains originated from France, Zeaiter et al. identified 4 genotypes grouped into 2 lineages, Marseille, including genotypes Marseille and CAL-1, and Houston-1, including genotypes Houston-1 and ZF-1; this remains the largest genetic

study of *B. henselae* human strains (*24*). Of the 107 human strains, 63 and 40 hold genotypes CAL-1 and ZF-1, respectively, which were predominant among French human strains (*24*). In our study, among 73 French human strains, 14 MST genotypes were also identified into 2 lineages, Houston-1, including 38 strains of 2 genotypes, and lineage Marseille, including 35 strains of 12 genotypes; genotypes 22 and 5 within lineages Houston-1 and Marseille, respectively, contained 37 and 22 human strains and were predominant. Thus, the phylogenetic relationships of French human strains identified by *pap31* were similar to what was found by using MST, although MST was more discriminatory than *pap31*-typing ($p<0.01$). However, MLST based on 9 genes later identified 7 genotypes and 3 lineages (Marseille, Houston-1, and Berlin-2) among 20 cat and 17 human isolates (*27*). In contrast, our results, which were based on a larger number and a wider distribution of both cat and human strains and the more discriminatory MST, differ from those of these and other authors in that we identified 2 lineages (lineages 1 and 4) besides Marseille and Houston-1 (*18*,*27*). Lineage 1 contained only Asian and American strains, which were not included in the study by MLST. Lineages Marseille and Houston-1 thus appear to be the main phylogenetic organization of *B. henselae* species. However, the phylogenetic organization of *B. henselae* species, as shown by both MLST and MST, was more complex than the structure of 2 main lineages (*27*,*31*). The 4 lineages based on MST provided the most detailed and reasonable illustration of the phylogenetic organization of *B. henselae* species because of its ability to show the geographic distribution of *B. henselae*. However, more strains, especially more human strains of various origin, should be studied by using MST to verify and modify this phylogenetic organization.

When we compared the MST classification to classification by 16S rRNA genotypes, we observed that 16SrRNA type I strains were restricted to clusters 1 and 2 (Houston-1), whereas most type II strains were grouped into clusters 3 (Marseille) and 4. However, a discrepancy between MST and 16S rRNA typing was observed within the Houston-1 cluster. One German cat strain classified in MST genotype 22 exhibited the 16S rRNA type II, in contrast to all other strains for which the 16S rRNA type was known, including other German strains. Such a discrepancy is consistent with the findings of Iredell et al., who demonstrated that 16S rRNA typing of *B. henselae* isolates was not entirely congruent with their lineage allocations (*27*).

In conclusion, we demonstrated, with 16 genotypes identified among 75 *B. henselae* human strains, that MST was more discriminatory than previously described methods for investigating *B. henselae* infection in humans. We did not find any statistically significant difference in genetic diversity between human and cat isolates of *B. henselae*. The studied human strains, although geographically limited, were phylogenetically organized into 2 clusters, which matched the origin of cat strains previously described as Houston-1 and Marseille clusters. Further studies incorporating strains from more diverse geographic origins and clinical features will be needed to improve our understanding of the population dynamics of *B. henselae.* We believe that MST can be a valuable tool for this purpose.

Dr Li is a physician and research scientist at the World Health Organization Collaborative Unité des Rickettsies in Marseille, France. His main research interests are the epidemiology and genetic diversity of human pathogenic bacteria.

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Human Noroviruses in Swine and Cattle

Kirsten Mattison,* Anu Shukla,* Angela Cook,† Frank Pollari,† Robert Friendship,‡ David Kelton,‡ Sabah Bidawid,* and Jeffrey M. Farber*

Human noroviruses are the predominant cause of foodborne gastroenteritis worldwide. Strains of norovirus also exist that are uniquely associated with animals; their contribution to the incidence of human illness remains unclear. We tested animal fecal samples and identified GIII (bovine), GII.18 (swine), and GII.4 (human) norovirus sequences, demonstrating for the first time, to our knowledge, that GII.4-like strains can be present in livestock. In addition, we detected GII.4-like noroviral RNA from a retail meat sample. This finding highlights a possible route for indirect zoonotic transmission of noroviruses through the food chain.

Noroviruses (NoVs) are a widespread cause of nonbacterial gastroenteritis (*1*,*2*). These agents are highly infectious, and person-to-person contact, fomites, and food have all been implicated in their spread (*3*). NoVs infect hosts of all ages and can cause very large outbreaks in closed settings. For example, observations from cruise ships demonstrate that infection has spread rapidly (*4*).

Since the *Norovirus* genus comprises viruses that infect humans, pigs, cattle, and mice, the possibility for zoonotic transmission of infection exists. In general, zoonotic transfer could occur either indirectly through the food chain or directly through animal contact. Transfer of animal virus to humans may produce an infection more serious than that traditionally associated with NoV (*5*). High-profile examples of zoonotic viruses transmitted to humans include the highly pathogenic avian influenza virus, known as H5N1 (*6*), and the severe acute respiratory syndrome (SARS) coronavirus (*7*). If NoVs have the potential for zoonotic transmission, monitoring emerging viral strains and tracking their virulence characteristics will be important to limit the potential for negative public health effects from any zoonotic transfer event.

NoVs are nonenveloped, polyadenylated, single-stranded, positive-sense RNA viruses of the family *Caliciviridae* (*8*). The noroviral genome is 7.3–7.7 kb in length and contains 3 open reading frames (ORFs) (*9*). ORF1 encodes a polyprotein that is autocatalytically cleaved to produce an N terminal protein, the nucleoside triphosphatase, a 3A-like protein, the viral VpG, a 3C-like protease, and the RNA-dependent RNA-polymerase (*10*,*11*). ORF2 encodes the major capsid protein (*12*), and ORF3 codes for a minor structural protein (*13*). NoV strains are currently classified according to alignment of the amino acid sequence for the major capsid protein. This classification system divides the known NoV into 5 genogroups (*14*). Within these genogroups, 31 genetic clusters have been defined (*14*,*15*). Genogroup GI, GII, and GIV viruses infect humans; GIII NoV infect cattle; and GV NoVs infect mice (*16*,*17*). To date, NoVs detected in naturally infected pigs belong to GII (*15*,*18*). The porcine and human viruses belong to different clusters within GII; porcine viruses are identified as GII.11, GII.18, and GII.19, and the human viruses make up the remaining 16 GII clusters (*15*,*18*).

Porcine NoV detected in Japan, the Netherlands, and the United States is closely related to the human NoV (*15*,*18*,*19*). Furthermore, human NoV can replicate and induce an immune response in gnotobiotic pigs (*20*), which indicates that swine could serve as a reservoir for human NoV. In addition, the genetic similarity between human and swine NoV strains raises the possibility that porcine-human recombinants could emerge during co-infection of the same host (*15*).

Cattle can also be infected with NoV; however, this strain is less closely related to the human NoV (*21*). Bovine NoVs produce recombinants in a similar manner as human

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NoVs (*22*). Thus, a cow co-infected with both bovine and human strains of NoV could conceivably produce a recombinant virus with altered virulence properties.

In this study, we tested fecal samples obtained from pig and dairy farms, as well as retail meat samples for the NoV genome. Swine (GII.18) and bovine (GIII) strains were detected in the swine and cattle samples, respectively. We also identified GII.4 (human) NoV sequences in both types of animal sample. This is the first report, to our knowledge, of GII.4-like NoV in animal fecal samples. Additionally, we found a GII.4-like strain of NoV associated with a retail raw pork sample.

These data suggest a potential mechanism for zoonotic transmission of NoV to humans through meat, dairy, or farm samples from infected pigs and cows. These findings also highlight the possibility that a recombinant swine/human or bovine/human NoV could emerge with altered tropism or virulence characteristics.

Materials and Methods

Swine fecal samples (N = 120) were collected in 3 sets from 10 farms in Canada farms. In September and October 2005, 4 composite samples were collected from each farm: 3 of fresh pen manure and 1 of stored pit manure. This sampling program was repeated in November and December of 2005 and again in April and May 2006. The fecal samples were placed into sterile containers, shipped overnight on ice, and stored frozen. Bovine fecal samples (N = 179) were collected from May to October 2006 from 45 different dairy farms in Canada. In this sampling program, farms were sampled only once each; 4 composite samples were taken at each farm, 1 each from calf pens (fresh), milking cow pens (fresh), heifer pens (fresh), and storage pits (stored). The dairy cattle samples were also placed into sterile containers, shipped overnight on ice, and stored frozen. A total of 156 retail meat samples were purchased, 15 at a time, during January–March 2006, and 6 at a time during July–November 2006. The last purchase consisted of only 4 samples. Each purchase was equally divided between samples of raw chicken, beef, and pork, except for the last, which had 1 extra chicken sample.

Fecal samples were suspended in 0.9% NaCl (5% wt/vol), vortexed briefly, and clarified through a combination glass fiber/PVDF 0.22-μm filter (Millipore, Mississauga, Ontario, Canada). RNA was extracted from a 140-μL sample of the resulting filtrate by using the QIAamp viral RNA extraction kit according to the manufacturer's recommendations (QIAGEN, Mississauga, Ontario, Canada). Total RNA was extracted from 25-g meat samples by using Tri reagent (Sigma, Oakville, Ontario, Canada) and Dynabeads coated with oligo-dT (Invitrogen, Burlington, Ontario, Canada) as described (*23*).

The RNA was used as a template for 1-step reverse-transcription–PCR (RT-PCR) using Monroe region B primers (*24*) or Ando region A primers (*25*) and the OneStep RT-PCR kit (QIAGEN). Amplicons of 213 bp and 123 bp were considered to be presumptively positive for region B and region A, respectively. They were gel purified by using a QIAquick gel extraction kit (QIAGEN) and sequenced on both strands by DNA Landmarks (St. Jean sur Richelieu, Québec, Canada). The primer sequences were removed, and the resulting 172 bp or 81 bp of sequence was aligned to NoV standard genotypes (G1.1 GenBank accession no. M87661, GI.2 no. L07418, GI.3 no. U04469, GI.4 no. AB042808, GI.6 no. AF093797, GII.1 no. U07611, GII.2 no. X81879, GII.3 no. U02030, GII.4 nos. X86557 and AY502023, GII.5 no. AF397156, GII.6 no. AB039776, GII.8 no. AB039780, GII.9 no. AY038599, GII.10 no. AF504671, GII.11 no. AB074893, GII.12 no. AB045603, GII.16 no. AY772730, GII.17 no. AY502009, GII.18 no. AY823305, G3.1 no. AJ011099, G3.2 no. AF097917, G4.1 no. AF414426, G5.1 no. AY228235 [*14*,*15*]) using ClustalW (*26*). The alignments were subjected to phylogenetic analysis with 2,000 replicates for bootstrapping by using the Seqboot, Dnadist (F84), Neighbor, and Consense programs, as implemented in the PHYLIP package (*27*). Phylogenetic trees were generated by using TreeView software (*28*).

Results and Discussion

NoV Detection in Swine Fecal Samples

A total of 120 swine fecal samples were tested for NoV RNA by using region B primers (*24*); of these, 30 were confirmed by sequence analysis to contain partial genomic sequence from NoV. Detecting NoV RNA from 25% of swine fecal samples tested in this study contrasts with the results of other surveys, in which the rates observed were 2% in US adult swine (*15*), 20% in US finisher pigs (older, heavier pigs being finished before slaughter) (*29*), and 0% in Venezuelan pigs (*30*). The different specificities of the primer sets used in the 4 studies likely account for the differences in virus recovery.

The sequence of the NoV polymerase region amplified was determined for the 30 strains and compared with those of reference strains. According to the region B sequence analysis, 3 different genotypes of NoV were detected in the Canadian pig samples (Figure 1), belonging to the swine GII.11 cluster (*18*), the swine GII.18 cluster (*15*), and the human GII.4 (Farmington Hills) cluster (*14*) (Figure 1). There were 22 strains of the swine GII.18 type, 6 of the swine GII.11 type, and 4 of the human GII.4 type; 2 samples contained both a swine GII.11 and a swine GII.18 virus. Sequences on both strands for the entire 172-bp region

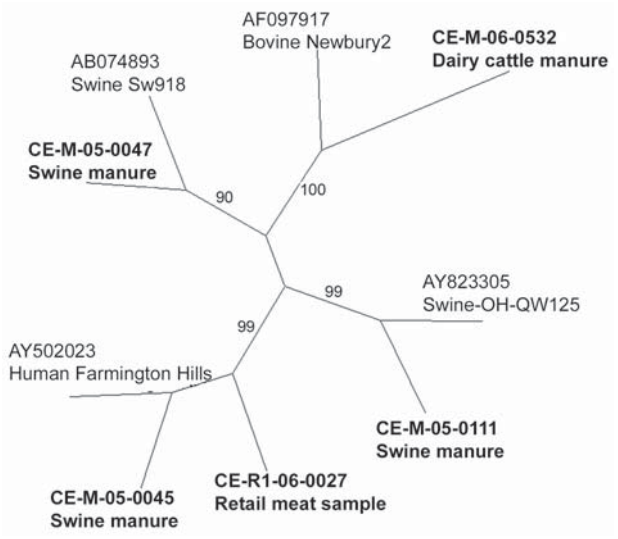

Figure 1. Unrooted neighbor-joining phylogenetic tree of representative noroviral strains and reference strains based on 172 bp of the RNA-dependent RNA polymerase region. GenBank accession nos. are indicated for the 5 reference strains (plain type), and the C-EnterNet sample codes are indicated for the representative strains identified in this study (**boldface type**). Bootstrap values are shown as percentages along the central branches

were obtained for 11 swine GII.18 viruses, 1 swine GII.11 virus, and 4 human GII.4 viruses. The 11 swine GII.18 strains had pairwise nucleotide identities ranging from 82% to 99% with no 2 identical strains (data not shown). Sequences from 2 of the 4 detected human strains were identical to each other and to the Farmington Hills reference strain (CE-M-05–0045 and CE-M-05–0102, Figure 2). The other 2 human NoV sequences amplified from swine manure were not identical to any other known sequences (CE-M-06–0013 and CE-M-05–0114, Figure 2). None of the amplicons sequenced were identical to the laboratory strains that we routinely use in our research activities (BMH-06–001 and BCCDC-04–684, Figure 2). Reasons for the incomplete sequencing of the remaining strains include low concentrations of RNA and overlapping peaks in portions of the chromatograms. A single representative of each group is shown in Figure 1 for clarity.

These results raise 2 possible issues related to public health. The first is that GII.4 NoV may be transferred directly from pigs to humans or from humans to pigs, providing a zoonotic source for human NoV outbreaks and a human source for pig NoV outbreaks. Second, pigs may be co-infected with both a human and a swine strain of NoV, potentially leading to recombination and generation of a new NoV strain with altered virulence properties. We did not identify GII.18-like and GII.4-like NoVs from the same manure sample; however, on 3 separate occasions, we detected both a GII.4-like and a GII.18-like NoV from the same farm on the same sampling date. A new variant of NoV with increased virulence has been seen in the GII.4 cluster in the past (*31*,*32*).

NoV Detection in Bovine Fecal Samples

Of the 179 bovine fecal samples tested for NoV RNA with region B primers (*24*), 3 were confirmed by sequence analysis to contain NoV genomes. Thus, we detected NoV RNA in 1.6% of bovine fecal samples tested in this study, in contrast to other surveys, in which the rates were 72% of US veal calves (*33*) and 4% of Dutch dairy cattle specimens (*34*). This finding likely reflects the specificity of the primer sets used, although it may also relate to the type of animal tested.

The sequence for region B of the NoV polymerase was determined for these NoV strains and compared with reference strains. Based on this sequence, 2 different genotypes of NoV were detected in the Canadian dairy farm samples, belonging to the bovine GIII.2 Newbury cluster and to the human GII.4 Farmington Hills cluster. The complete 172-bp region B sequence was confirmed only for the sequence from CE-M-06–0509; this sequence was not identical to any of the other detected GII.4 sequences (Figure 2).

We also obtained an amplicon representing region A of the NoV polymerase from CE-M-06–0509. By region B phylogeny, the strain was classified as GII.4 (Farmington Hills) NoV (CE-M-06–0509; Figures 1, 2). Interestingly, the region A sequence did not share >77% identity with any of the NoV strains in the GenBank database. Phylogenetic comparisons to the standard viruses described by Zheng et al. (*14*) were not successful in assigning a genogroup and cluster for this sequence; bootstrap values were 16%–40% for most branches in the tree (data not shown). A large grouping of strains did emerge in this analysis, showing that in region A, this NoV strain was more closely related to GII.1, GII.2, GII.3, GII.4, GII.5, GII.10, GII.12, and GIII.1 sequences than to the other reference strains (data not shown). This finding indicates that the viruses detected and characterized as GII.4 NoV by using region B primers from swine and bovine manure (Figures 1 and 2) may represent novel NoV types, with genetic content slightly different from the previously recognized human, swine, and bovine-type species. This finding would also explain the difficulty we have had in obtaining region A, C, and D amplicons from the other manure samples (data not shown).

Human NoVs have not been shown experimentally to infect cattle; however, the human and bovine NoVs share a cross-reactive epitope in their capsid proteins (*35*). The risk for bovine NoV infection in humans is thought to be low (*21*). Co-infection of an animal with both human and bovine NoV may result in a recombinant agent with altered virulence, as the bovine NoVs have been shown to undergo

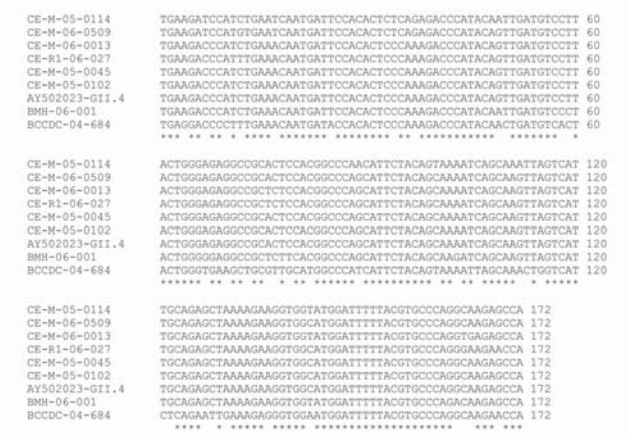

Figure 2. Nucleotide alignment of the 172-bp amplified region from the human GII.4-like strains. Four were detected in swine manure (CE-M-05–0114, CE-M-06–0013, CE-M-05–0045, and CE-M-05–0102), 1 in bovine manure (CE-M-06–0509), and 1 in a retail meat sample (CE-R1–06–027). The reference sequences provided are from the Farmington Hills reference strain (GenBank accession no. AY502023) and our laboratory strains, BMH-06–001 and BCCDC-04–684. Asterisks indicate identity at this position among all strains.

extensive genetic recombination in a similar manner to the human viruses (*36*–*39*).

NoV Detection in Retail Raw Meat Samples

To date, 156 retail meat samples have been processed and tested for the presence of NoV. One sample of raw pork has tested positive for a NoV of the GII.4 cluster (CE-R1–06–0027; Figures 1, 2). The region B sequence for this strain was not identical to any of the other sequences identified in this study (Figure 2). While we cannot determine the source of the contamination (i.e., at slaughter, during handling, or during packaging), our findings demonstrate that retail meat is a potential route for the indirect zoonotic transmission of NoV. This finding highlights the importance of proper handling and cooking for meat products. NoV titer is reduced by 4 orders of magnitude in <1 min at 71°C (*40*), the temperature which Health Canada recommends for the cooking of pork chops.

Conclusion

This is the first report, to our knowledge, of GII.4-like NoV sequences in animal fecal samples. The GII.4 NoV sequences were all detected in fresh manure samples taken from animal pens, which limits the chances that they were derived from an unknown source of human waste. They were sampled from different farms on different days, and results from all processing and PCR control samples were negative. The use of the human-specific region B primer set (*24*) in our study allowed the identification of human NoV from animal specimens, in contrast to previous surveys,in which swine or bovine-specific RT-PCR primers were used (*15*,*18*,*19*,*34*).

Although GII.4 viruses can infect piglets experimentally (*20*), these data are the first indication that such infections may occur naturally as well. We have also identified partial GII.4 NoV genomic sequences for the first time in cattle feces. Retail samples have not been previously tested as part of a surveillance scheme for the presence of NoV. Our findings support previous suggestions that NoV may have a zoonotic route of transmission to humans (*15*). It follows that infected humans may pass the virus to livestock as well, and some outbreaks of gastroenteritis in farm animals may be prevented by controlling the access of ill workers to livestock. Our results also highlight the possibility that a recombinant swine/human or bovine/human NoV could emerge with altered tropism or virulence characteristics. In conclusion, we stress the importance of monitoring existing and emerging NoV strains to mitigate the potential impact of a recombinant NoV's being transmitted to the human population from either a swine or a bovine source.

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Dr Mattison is a research scientist in food virology with the Bureau of Microbial Hazards, Food Directorate, Health Products and Food Branch, Health Canada. Her research involves the detection, epidemiology, and inactivation of foodborne viruses, including NoVs.

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High Prevalence of Tuberculosis in Previously Treated Patients, Cape Town, South Africa

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The tuberculosis (TB) notification rate is high and increasing in 2 communities in Cape Town, South Africa. In 2002, we conducted a prevalence survey among adults ≥15 years of age to determine the TB prevalence rate; 15% of households in these communities were randomly sampled. All persons living in sampled households were eligible for chest radiography and sputum examination. Of the 3,483 adults who completed a questionnaire, 2,608 underwent chest radiography and sputum examination. We detected 26 bacteriologically confirmed TB cases and a prevalence of 10.0/1,000 (95% confidence interval [CI] 6.2–13.8 per 1,000). We found 18 patients with smear-positive TB, of whom 8 were new patients (3.1/1,000, 95% CI 0.9–5.1/1,000). More than half of patients with smear-positive TB (10, 56%) had previously been treated. Such patients may contribute to transmission of *Mycobacterium tuberculosis* and the high TB prevalence rate. Successful treatment of TB patients must be a priority.

In 2003, South Africa had an estimated incidence of 218 new smear-positive tuberculosis (TB) cases per 100,000 population. This country ranked eighth in the world for total number of TB cases per country and tenth for incidence rates (*1*). Western Cape Province had the highest notification rate in this country (*2*). In Cape Town, the largest city in this province, the notification rate for new smear-positive TB was 266/100,000 in 2002 (*3*). In Ravensmead and Uitsig, 2 neighboring urban communities in Cape Town, the rate of registered new smear-positive TB cases increased from 228/100,000 in 1994 to 299/100,000 in 1998 and to 341/100,000 in 2002 (*4*,*5*).

Although improved case detection may account for these increases, a true increase in incidence may also be occurring. Currently, there is no reliable estimate of the TB situation in this region. Furthermore, no data are available on number of undetected cases in the 2 communities, care-seeking behavior and delay in diagnosis, and number of persons who previously had TB, all of which are factors that may contribute to transmission of TB. We suspect that this population contains a substantial number of TB patients who previously had this disease, and that this group contributes to transmission of TB and the persistent high rates of TB notification.

The aim of this TB prevalence survey was to obtain a reliable estimate of the situation, and to determine the number of undetected cases in the 2 communities, the number of persons who have previously had TB, and the proportion of previously treated cases among undetected TB patients. Results of this study will be used to plan interventions to control the TB epidemic in these 2 communities in Cape Town.

Methods

Study Area

We conducted a TB prevalence survey in the communities of Ravensmead and Uitsig, which have a total surface area of 3.5 km^2. In 2001, the study area had a population of 36,334 (*5*). Two primary healthcare clinics and an adjacent tertiary care hospital serve the area. The World Health

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Organization directly observed treatment short-course strategy was introduced in these 2 communities in 1997.

Study Design

The TB prevalence survey was part of a larger community survey, the Lung Health Survey. In addition to prevalence of TB, the Lung Health Survey aimed to determine the prevalence of lung diseases, including asthma and chronic obstructive pulmonary disease. The current report deals only with TB. Information on other lung diseases will be reported elsewhere.

The study included 2 neighborhoods with similar socioeconomic status. We selected a random sample of all addresses in the study area and defined an address as the residential geographic location (either a physical street address or the name and number of a flat). A randomized 15% sample was taken from all residential addresses. A total of 5,592 addresses were situated in the study area, of which 839 households were selected for participation in the survey. The study protocol was reviewed and approved by the ethics committees of Stellenbosch University and the University of Cape Town.

Survey Procedures

The Lung Health Survey was conducted from July 1 through December 15, 2002. Trained community workers counted the number of persons at each selected address. All persons at selected addresses (including the main house and all backyard shacks) were eligible for investigation. If the head of the household did not give consent for the household to be enrolled in the survey, the household was replaced with a household at an adjacent address, e.g., first to the right and then to the left of the household that declined participation. All persons living at the address were informed of the purpose of the survey, and written informed consent was obtained from each participant before enrollment. The Lung Health Survey included both adults and children, but here we report data in our survey only for adults ≥15 years of age.

All participants, supervised by a trained field worker, completed a questionnaire containing questions on demographic characteristics and earlier TB treatment. Patient category and treatment outcome of previous disease episode(s) were obtained from the local healthcare center.

Participants were then transported to a nearby facility where chest radiographs (35 × 43 cm postero-anterior view) were performed by using a 200-mA chest radiography machine. Within 1 week, a pulmonologist screened the radiographs for abnormalities. Persons with abnormalities that required immediate investigation or treatment were referred to a local hospital. An accredited reader assessed all radiographs when the survey was finished by using a standardized classification system developed for epidemiologic surveys (*6*). Abnormalities were classified as being either consistent with TB or not related to TB. Parenchymal, pleural, and central structure abnormalities were considered consistent with TB. A second experienced reader then reread a stratified sample of 31% of the radiographs. Kappa agreement between the 2 readers was 0.69 (95% confidence interval [CI] 0.64–0.74) for abnormalities consistent with TB and 0.47 (95% CI 0.42–0.53) for whether the radiographic result was normal (*6*). On the basis of these results, a second reader was deemed unnecessary.

Upon request, each participant provided a sputum specimen at the health center where the chest radiograph was obtained by using the active cycle of breathing technique (*7*). Specimens were collected in a wide-mouthed plastic container with a secure screw-top lid. This container was then transported to the laboratory where it was processed within 3 days. A second specimen was obtained from persons who had a positive smear, a positive culture, or both, before they were referred for treatment to a nearby clinic.

Laboratory Procedures

One smear and 1 culture were prepared from each sputum specimen. Smears were stained by using the Ziehl-Nielsen technique with carbol fuchsin and methylene blue. The smears were scored by 1 reader according to the guidelines of the International Union Against Tuberculosis and Lung Disease (*8*). A smear result was considered positive if we observed ≥1 acid-fast bacillus per 100 oil-immersion fields. This provision included scanty smears because these are considered indicative of true positivity (*9*). Sputum samples were liquefied and decontaminated with 4% NaOH by using standard procedures (*10*). Samples were then centrifuged at 3,000 × *g* for 15 minutes. Concentrated decontaminated sputum sediment was resuspended in 2 mL phosphate buffer (68 mmol/L, pH 6.8) and placed on Löwenstein-Jensen slants. These slants were then incubated at 37°C for 6 weeks.

Definitions, Data Processing, and Statistical Analysis

A bacteriologically confirmed case of TB was defined as a person with either 2 positive smears or 2 positive cultures, or 1 positive smear and 1 positive culture. Participants with 1 positive smear or 1 positive culture whose chest radiographs showed TB-related abnormalities, or those with a positive sputum smear or culture results from specimens collected at the health center within 2 months after sputum collection in the prevalence survey, were also considered to have bacteriologically confirmed TB.

Data were entered into a Microsoft (Redmond, WA, USA) Access database. Inconsistencies between the 2 entries were checked against original data and errors were corrected. Statistical analysis was performed by using SPSS version 12.0.1 for Windows (SPSS Inc., Chicago,

IL, USA) and STATA version 8.0 for Windows (*11*). Prevalence of TB was calculated by dividing the number of TB cases by the number of participants who attempted to provide a sputum specimen. Those who were unable to provide a sputum specimen were considered smear-negative and culture-negative. Results include crude prevalence rates and prevalence rates adjusted for the cluster sampling design. Households served as the primary sampling units and variation at this level was taken into account.

We calculated prevalence after applying a correction factor for 100% collection coverage. Results were weighted to adjust for unequal coverage in the different sex and age groups (15–34 and ≥35 years of age). Separate weighting was applied to participants in the original and replacement samples. We calculated the sampling weight as the product of (1/selection probability of a household) and (1/participation rate). The selection probability of a household was 0.15 because we randomly sampled 15% of the households. The participation rate varied by group sampled; e.g., 68% of women 15–34 years of age in the original sample had a chest radiograph performed. Sampling weight was (1/0.15) × (1/0.68) = 9.8. CIs were calculated by using the normal approximation to the binomial distribution.

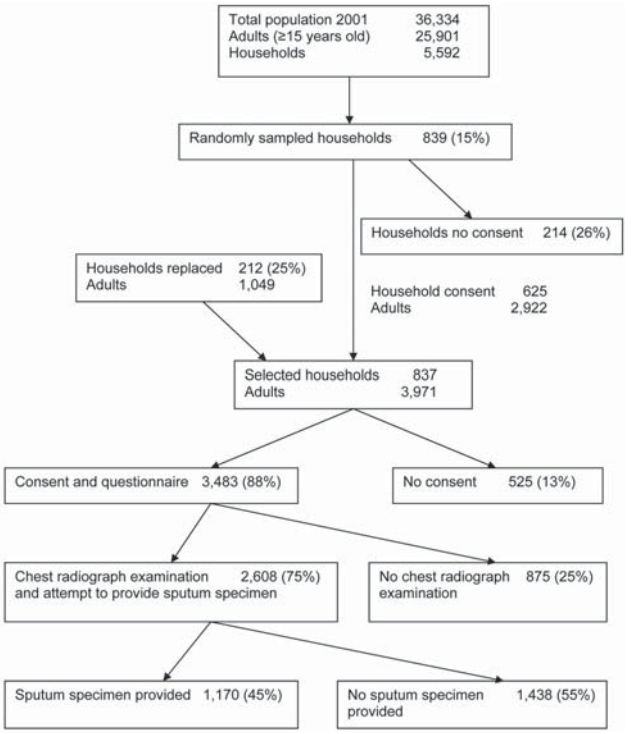

Figure. Sample selection of 3,483 adults, Cape Town, South Africa, for the study.

Results

Study Population and Coverage of Measurements

Initially, heads of 625 (74%) of 839 households consented to participate in the study. We replaced 212 of 214 households that refused participation; 2 households could not be replaced. Of 214 nonparticipating households, 81 provided demographic details of persons living in the household. Occupants of these 81 households did not differ from the 212 households that were resampled with respect to sex (odds ratio [OR] 1.00, 95% CI 0.82–1.23) or age (t = 0.33, p = 0.74, by Student *t* test). In the final sample, residents within 837 households included 3,971 adults, of whom 3,483 (88%) consented and completed a questionnaire (Figure). Of these, 2,608 (75%) had a chest radiograph and attempted to provide a sputum specimen. Persons ≥35 years of age (78%) had a chest radiograph more often than persons <35 years of age (72%) (OR 1.41, 95% CI 1.21–1.65). More women (78%) than men (71%) had a chest radiograph (OR 1.48, 95% CI 1.26–1.72). Of those who had a chest radiograph, 1,170 (45%) were able to provide a sputum specimen. Thirteen specimens were contaminated or inadequate for culture because of insufficient volume.

Prevalence of TB

Of 2,608 participants with chest radiographs, 702 (26.9%) had an abnormality on the radiograph (95% CI 25.2%–28.6%). In 337 (12.9%) of these radiographs, abnormalities were consistent with TB (95% CI 11.6%–14.2%) (Table 1). Prevalence of TB-related abnormalities on chest radiographs increased with age (χ^2 for linear trend 59.1, p<0.001) and were more often seen in men (16%) than in women (11%) (OR 1.48, 95% CI 1.16–1.89).

Twenty-nine participants had a positive smear or positive culture, of whom 3 did not fulfill the definition of a bacteriologically confirmed TB case; 2 had a scanty smear and 1 had a positive culture. The remaining 26 participants fulfilled the definition of bacteriologically confirmed TB, giving a prevalence of 10.0/1,000 (95% CI 6.2–13.8/1,000) (Table 1). Of these 26 patients with bacteriologically confirmed TB, 16 (62%) were new patients (no previous treatment), giving a prevalence of new bacteriologically confirmed TB cases of 6.1/1,000 (95% CI 3.1–9.1/1,000). Ten (56%) of the 18 smear-positive patients had been previously treated for TB. The prevalence of previously treated smear-positive TB cases was 3.8/1,000 (95% CI 1.5–6.2/1,000). Only 8 (44%) smear-positive cases were new cases, and the prevalence of new smear-positive TB cases was 3.1/1,000 (95% CI 0.9–5.1/1,000).

Correction for the cluster sampling design and adjustment for noncoverage had little effect on the prevalence estimates (Table 1). Three of the 26 patients with bacteriologically confirmed TB were receiving anti-TB therapy at the time of the survey. Nineteen of the remaining 23 pa-

Table 1. Prevalence estimates of tuberculosis (TB) in adults ≥15 years of age, Cape Town, South Africa*

Characteristic	No. positive/ no. tested	Crude prevalence estimate	Prevalence estimate corrected for simple random sampling at household level	Weighted prevalence estimate, corrected for sampling and nonresponse
History of TB (questionnaire)	338/3,483	9.7% (8.7%–10.7%)	9.7% (8.6%–10.8%)	9.8% (8.7%–10.9%)
TB abnormalities on chest radiograph	337/2,608	12.9% (11.6%–14.2%)	12.9% (11.6%–14.3%)	12.9% (11.5%–14.3%)
Total smear-confirmed TB	18/2,608	6.9 (3.7–10.1)	6.9 (3.3–10.5)	7.1 (3.3–10.8)
New smear-confirmed TB	8/2,608	3.1 (0.9–5.2)	3.1 (0.9–5.2)	3.0 (0.9–5.1)
Total bacteriologically confirmed TB	26/2,608	10.0 (6.2–13.8)	10.0 (5.7–14.3)	10.2 (5.8–14.6)
New bacteriologically confirmed TB	16/2,608	6.1 (3.1–9.1)	6.1 (3.0–9.3)	6.2 (3.0–9.3)

*Prevalence estimates are per 1,000 unless otherwise stated (percentage values). Values in parentheses are 95% confidence intervals.

tients with detected cases began treatment <6 months after these cases were detected in the survey. Of those patients, 12 were cured, 1 completed treatment without confirmed smear conversion, 2 interrupted treatment, 1 died, and 3 had an unknown outcome.

History of TB

All 10 patients with bacteriologically confirmed TB who had previously received treatment for TB were smear-positive. Four of the 10 previously treated patients had been considered cured during their previous disease episodes, and 3 had interrupted treatment before completion. For 3 previously treated smear-positive patients, no information was available on the outcome of their previous disease episode (Table 2). Four patients had been treated for TB on more than 1 occasion before their cases were detected in the survey. Also, 3 previously treated patients lived in the same household. Bacteria isolated from 1 of 7 previously treated TB patients who underwent drug sensitivity testing were resistant to isoniazid (Table 2).

A history of TB was reported by 338 (9.7%) of 3,483 adults (95% CI 8.7%–10.7%) (Table 1). The prevalence of bacteriologically confirmed TB was 29.9/1,000 (95% CI 11.4–47.7/1,000) in those who were previously treated for TB compared with 5.1/1,000 (95% CI 2.6–7.6/1,000) for persons who had never had TB (OR 5.96, 95% CI 2.68–13.25). Participants reporting a history of TB were significantly older (mean 41 years) than participants who had never had TB (mean 38 years) ($t = 2.59$, $p = 0.01$, by Student t test). A history of TB was more common in men (12%) than in women (8%) (OR 1.60, 95% CI 1.27–2.01).

Discussion

The prevalence of bacteriologically confirmed TB in our study was high, and the proportion of patients that had ≥1 previous episode of TB was substantial. These data support the hypothesis that previously treated cases form a large proportion of TB patients in the 2 communities. Therefore, persons with previously treated TB are likely to contribute to transmission of *Mycobacterium tuberculosis*, and may also be a factor in maintaining high prevalence rates of TB.

Low treatment success rates may partly explain the high proportion of previously treated cases. Treatment success rates for new smear-positive cases were 78% in 2001 and 80% in 2002 (*4*), which were lower than the 85% recommended by the World Health Organization (*1*). Low cure rates in TB patients may lead to reactivation of this disease and could also result in patients with chronic TB who excrete TB bacilli over an extended period. However, recent studies using DNA fingerprinting showed that a large proportion of recurrent TB in our study area is caused by exogenous reinfection rather than reactivation (*12*,*13*). Approximately 10% of the survey population had previously had TB, as shown by responses to the question of whether persons had ever had TB and by TB-related abnormalities on chest radiographs. Previously treated persons had a much higher prevalence rate of TB than persons who had never had TB. Achieving high cure rates and preventing TB patients from infecting other people must therefore be considered the highest priority.

The high proportion of previously treated undetected cases suggests that case detection for previously treated cases is insufficient. A case-detection rate of 118% was reported for new smear-positive cases in South Africa (*1*), but no information on previously treated cases has been reported. Further research is needed to determine the case-detection rate for previously treated patients.

Previously treated TB is associated with multidrug-resistant TB and may be associated with extensively drug-resistant TB. A survey on drug resistance conducted in 1992–1993 in the Western Cape Province showed a rate of 8.6% acquired and 3.2% initial drug resistance in Cape Town (*14*). Recently, a study among hospitalized children (<13 years of age) showed an increase in isoniazid resistance from 6.9% in 1994–1998 to 12.8% in 2003–2005

Table 2. Treatment history of 10 previously treated tuberculosis (TB) patients with sputum smear–positive TB test results, Cape Town, South Africa*

Patient no.	Patient category	Year of previous episode	Outcome	Drug resistance
1	New	1985	Cured	NT
	Retreatment after cure	1987	Cured	NT
	Retreatment after cure	1994	Cured	Sensitive
	Retreatment after cure	2000	Cured	Sensitive
	Retreatment after cure	2002	Cured	Sensitive
2	New	1996	Cured	Sensitive (2002)
3	New	1999	Cured	Sensitive (2002)
4	New	2001	Cured	Sensitive (2002)
5	New	1995	Interrupted	NT
6	Retreatment	1995	Interrupted	Sensitive
	Retreatment after interruption	1998	Failed	Sensitive
7	Retreatment	1997	Interrupted	INH resistant (2002)
8	Retreatment	1997	Unknown	NT
9	Unknown	Unknown	Unknown	NT
10	Unknown	Unknown	Unknown	Sensitive (2002)

*NT, not tested; INH, isoniazid.

(*15*,*16*). Drug-resistant TB is probably not the driving force behind the high prevalence of TB in the 2 study communities; however, it is likely to become an increasingly important factor.

Similarly, an increasing prevalence of HIV may be partly responsible for the increasing notification rates for TB, but does not adequately explain the high prevalence of TB in this study (*17*). HIV prevalence in the study area is not known and was not measured in this study. However, we believe that the prevalence rate for HIV in the study area is less than the rate of 12.4% in women attending public antenatal clinics in Cape Town in 2002 (*18*). Approximately 6% of patients with newly diagnosed TB in Ravensmead and Uitsig are HIV positive.

The high prevalence rates of TB in Ravensmead and Uitsig are reminiscent of similar observations in Inuit communities in Greenland, Canada, and the United States. In the Inuit population, an average annual incidence of active TB of 1,310/100,000 was reported from 1967 through 1969 (*19*). The highest rates of TB were reported in persons who had previously had TB (*20*), which was also observed in our study.

Our study was limited by a sample size that was insufficient to give precise prevalence estimates for bacteriologically confirmed and smear-positive TB. This resulted in wide CIs around the prevalence estimate (95% CI 1–6/1,000 for confirmed new smear-positive TB). Sampling bias due to resampling was minimized by replacing households that refused participation with generally similar neighboring households. For households that refused participation but provided some basic information on household members, no age or sex differences were found. For households that did not provide any information and refused participation, age or sex differences could not be tested.

Participants who were unable to provide a sputum specimen were considered to be smear negative and culture negative. This approach may have resulted in low prevalence estimates because some cases could have been missed. Okutan et al. (*21*) reported positive smears in 61% and positive cultures in 31% of gastric lavage specimens obtained from patients with suspected TB who were unable to provide sputum specimens. However, because the participants in our survey represented the entire population, not just those with suspected cases of TB, we anticipate that the proportion of missed cases will be considerably lower. The strength of our study was that sputum smear and culture was attempted for all participants. This differs from most surveys in which screening of symptoms, results of chest radiography, or both, are used as the basis for deciding which sputum specimen should be examined (*22–27*).

In conclusion, this prevalence survey supports the hypothesis that the prevalence of TB is extremely high in the area studied. Because previously treated smear-positive TB patients constituted more than half of the patients with prevalent smear-positive cases, the survey also suggests that these cases contribute to transmission of *M. tuberculosis*. Successful treatment of TB cases must be a priority in South Africa.

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Skin and Soft Tissue Infections Caused by Methicillin-Resistant *Staphylococcus aureus* USA300 Clone

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Until recently, methicillin-resistant *Staphylococcus aureus* (MRSA) has caused predominantly healthcare-associated infections. We studied MRSA infections and overall skin and soft tissue infections (SSTIs) in outpatients receiving care at the Baltimore Veterans Affairs Medical Center Emergency Care Service during 2001–2005. We found an increase in MRSA infections, from 0.2 to 5.9 per 1,000 visits (p<0.01); most were community-associated SSTIs. Molecular typing showed that >80% of MRSA infections were caused by USA300. In addition, SSTI visits increased from 20 to 61 per 1,000 visits (p<0.01). The proportion of SSTI cultures that yielded MRSA increased from 4% to 42% (p<0.01), while the proportion that yielded methicillin-sensitive *S. aureus* remained the same (10% to 13%, p = 0.5). The increase in community-associated MRSA infections and the overall increase in SSTIs in our population suggest that USA300 is becoming more virulent and has a greater propensity to cause SSTIs.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a cause of predominantly nosocomial or healthcare-associated infections. MRSA infections usually affect patients during hospitalization, after surgery, and during stays in long-term care facilities. In addition, MRSA infections are common in patients who have indwelling vascular catheters for dialysis or other medical treatments. However, during the past decade, multiple reports of community-associated MRSA (CA-MRSA) infections have been reported in patients who lack the above risk factors (*1*–*4*).

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CA-MRSA infections differ from healthcare-associated MRSA (HA-MRSA) infections in a number of ways. CA-MRSA infections are predominantly skin and soft tissue infections (SSTIs), are often susceptible to other non–β-lactam antimicrobial drugs, and carry a type IV or V staphylococcal cassette chromosome (SCC) with the *mec*A gene. In contrast, HA-MRSA infections are found at multiple sites, are usually multidrug resistant, and carry the SCC*mec* types I, II, and III (*5*,*6*). In the United States, 2 major clones of CA-MRSA have been identified by pulsed-field gel electrophoresis (PFGE) and named USA300 and USA400 by the Centers for Disease Control and Prevention (CDC) (*7*). Among the community-associated types, the USA300 clone has recently emerged as the predominant cause of SSTIs in the United States (*8*,*9*). Toxin expression between CA-MRSA and HA-MRSA strains differs. Most CA-MRSA strains carry the intracellular toxin Panton-Valentine leukocidin (PVL), which is known for pore formation on polymorphonuclear cells of the host (*10*,*11*). In addition, the USA300 clone contains the arginine catabolic mobile element (ACME), which inhibits polymorphonuclear cell production (*10*).

In the summer of 2004, physicians in our Emergency Care Service (ECS) alerted us to an increased number of outpatients who had SSTIs caused by MRSA. This observation led us to begin this investigation with the following objectives: 1) to measure the incidence of MRSA infections in our ECS, 2) to describe these infections and their isolates, and 3) to measure the incidence of SSTIs in our ECS and the entire associated healthcare system over the past 5 years. We present molecular and epidemiologic evidence that the emergence of the USA300 clone has led to

not only an increase in CA-MRSA infections but also an overall increase in SSTIs in our patient population.

Methods

Setting

The population for our retrospective study was derived from the Veterans Affairs Maryland Health Care System (VAMHCS), which provides comprehensive health care to >45,000 veterans in the Maryland area. Outpatient care is provided at 2 medical centers and 3 community-based outpatient clinics. Our ECS is located in the Baltimore VA Medical Center and serves ≈85 patients a day.

MRSA Infections

We reviewed microbiology cultures obtained during an ECS visit from October 1, 2000, through September 30, 2005 (fiscal years [FYs] 2001–2005), in which MRSA was first isolated from a patient's culture. The total number of ECS visits per FY was obtained from administrative records. The MRSA infection risk was calculated by dividing the total number of ECS patients whose culture results were MRSA positive for the first time by the number of ECS visits per FY. Information about patient demographics, clinical manifestations, and risk factors for nosocomial acquisition of MRSA was obtained from manual review of the VA's computerized patient record system. Patients were categorized as to site of infection as follows: SSTI (wound culture positive for MRSA in the setting of new erythema, induration, warmth or pain at that site), urinary tract infection (positive urine culture in addition to at least 1 sign or symptom of a urinary tract infection), and pneumonia (positive sputum culture for MRSA in addition to a new infiltrate on chest radiograph). The infection was classified as healthcare-associated if the patient had a history of hospitalization, surgery, dialysis, or had been a resident in a long-term care facility within 1 year before infection or had a percutaneous medical device or permanent indwelling catheter at the time of infection. An infection in a patient without these risk factors was categorized as community-associated (*12*).

MRSA Isolates

Clinical cultures were sent to the clinical microbiology laboratory of the VAMHCS. *S. aureus* was identified by following standard laboratory protocols. MRSA was defined as an *S. aureus* isolate that grew on oxacillin screen agar; methicillin-susceptible *S. aureus* (MSSA) was defined as an isolate that did not grow on oxacillin screen agar. Antimicrobial drug susceptibility was determined by following the methods and interpretation guidelines of the Clinical and Laboratory Standards Institute (*13*). Erythromycin-resistant and clindamycin-susceptible isolates were tested for inducible resistance by the D-test, following the guidelines that began on January 30, 2004. Clindamycin resistance data include all isolates that are truly resistant by breakpoint and isolates that have inducible resistance detected by the D-test. All isolates were frozen at –70°C in trypticase soy broth with 30% glycerol. MRSA isolates (n = 329) were typed by DNA sequencing analysis of the protein A (*spa*) gene hypervariable region as described (*14*). Allele identification was based on comparison with sequences in an *S. aureus* database (www.ridom.de/spaserver). PVL (*15*) and ACME (*10*) virulence factors were detected by following published protocols. PFGE was performed according to McDougal et al. (*7*). Photographic images of the gels were saved digitally with the Geldoc EQ (BioRad Laboratories, Hercules, CA, USA); gel analysis was saved with Fingerprinting II Software (BioRad Laboratories). The reference standard *S. aureus* NCTC 8325 was included in the first and fifteenth lane of each gel, and all isolates were normalized to this global standard. The band patterns were compared by means of the Dice coefficient by using the unweighted pair-group method to determine band similarity and following the criteria established by Tenover et al. to define the pulsed-field type clusters (*16*). We defined USA300 as isolates that had the MBQBLO repeat motif and were positive for PVL and ACME.

SSTIs

For SSTIs, the total number of ECS visits (n = 3,688) and VAMHCS visits (n = 13,041) per FY, according to codes from the International Classification of Disease, Clinical Modification 9 (ICD-9-CM) (680, carbuncle and furuncle; 681, cellulitis and abscess of finger and toe; 682, other cellulitis and abscess; 704.8, folliculitis), were obtained from administrative records. The rate of SSTIs was calculated for the ECS and the VAMHCS by dividing the number of total visits for SSTIs by the number of visits per FY for each site. We also measured the number of patients who had SSTIs by taking the first patient visit for each year. Finally, we assessed whether patients' infections were cultured during their visits for SSTI and whether that culture grew MRSA or MSSA.

Statistical Analysis

Rates were computed as previously mentioned. Proportions were used to describe categorical variables and means to describe continuous variables. Categorical variables were compared by using χ^2 or Fisher exact tests, as appropriate; continuous variables were compared by using Student *t* test. Statistical analysis was performed by using SPSS version 12.5 (SPSS Inc., Chicago, IL, USA).

Results

The proportion of ECS visits for MRSA infections for patients with no history of MRSA colonization or infec-

tion increased significantly from 0.2 per 1,000 ECS visits in FY01 to 5.9 per 1,000 visits in FY05 ($p<0.01$, χ^2 test; Figure 1). The absolute number rose from 6 in FY01 to 180 in FY05, and 280 (81%) of 329 cases occurred during FY04 and FY05. In FY01, only 3 SSTIs were caused by MRSA compared with 159 in FY05.

The mean age of patients with new MRSA infections (n = 329) was 56 years; 98% were male, 69% were African American, 84% had an SSTI, 8% had a urinary tract infection, 2% had pneumonia, and 6% had other sites of infection. Of these 329 MRSA isolates, 76% were susceptible to clindamycin, 85% to tetracycline (n = 257), and 97% to trimethoprim-sulfamethoxazole. Overall, 217 (66%) of the 329 patients with MRSA infections had no known contact with the healthcare system in the year before their infection and most likely acquired MRSA in the community.

Molecular typing was performed on 296 (90%) of the 329 MRSA isolates from these infections. *Spa* typing showed a single dominant clonal type with the MBQBLO repeat motif (Table 1). The proportion of isolates tested with this *spa* type group significantly increased from 0% in FY01 to 89% in FY05 ($p<0.01$, χ^2 test). Isolates that contained the virulence factors PVL and ACME also increased significantly from 0% in FY01 to 93% in FY04 and 89% in FY05 ($p<0.01$, χ^2 test) and strongly correlated with isolates of the MBQBLO repeat motif. Molecular studies showed that isolates defined as USA300 by having the MBQBLO repeat motif and being positive for PVL and ACME increased from 0% in FY01 to 84% in FY05 and that USA300 was the dominant clone in FY03–FY05.

To confirm that isolates of the *spa* clonal type that had the MBQBLO repeat motif and were positive for ACME and PVL represent USA300, we performed PFGE on a subset of the isolates (n = 31). This subset consisted of a random selection of 10% of the total isolates. Sixteen isolates were positive for USA300 by both typing methods: 1) PFGE and 2) containing the MBQBLO repeat motif, PVL, and ACME. Three isolates had PFGE types similar to USA300 and PVL but had neither the MBQBLO repeat motif nor ACME. Twelve of the isolates did not have PFGE or *spa* types similar to USA300. No isolate was positive for MBQBLO repeat motif, PVL, and ACME and negative for USA300 by PFGE. When the MBQBLO that were ACME and PVL positive were compared with PFGE patterns for USA300, the sensitivity was 84% and the specificity was 100%; positive predicted value was 100% and negative predicted value was 80%.

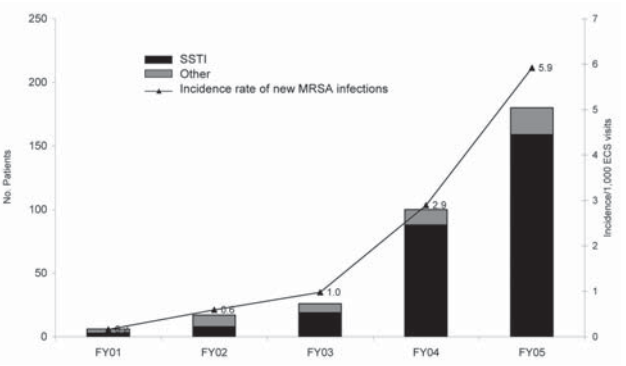

Figure 1. Methicillin-resistant *Staphylococcus aureus* (MRSA) infections in patients without a history of MRSA per 1,000 visits to the Baltimore Veterans Affairs Medical Center Emergency Care Service (ECS), 2001–2005. SSTI, skin and soft tissue infection; FY, fiscal year. FY01–03 versus FY04, χ^2 test, $p<0.001$.

Not all isolates that were USA300 according to PFGE correlated with the MBQBLO repeat motif and were positive for PVL and ACME. One isolate had an unrelated *spa* type (BQBPO repeat motif), and 2 isolates were negative for ACME. Eighteen of these isolates had the MBQBLO repeat motif, and PFGE showed a similarity to PFGE type USA300 (Figure 2). PFGE in our study determined that all of these related USA300 isolates carried PVL and all except 2 carried the ACME virulence factor. The 2 without ACME were closely related to SCC*mec* IVb type. However, of the overall 329 MRSA isolates, 5 had the MBQBLO repeat motif and were PVL positive but ACME negative. Four isolates had the MBQBLO repeat motif (*spa* type t064) but

Table 1. Molecular typing of isolates from patients with new MRSA infections, Baltimore Veterans Affairs Medical Center Emergency Care Service, 2001–2005*

Time period	No. isolates tested	*spa* typing			PVL, no. (%)	ACME, no. (%)	MBQBLO motif, PVL, and ACME, no. (%)
		MBQBLO motif,† no. (%)	MDMGMK motif,‡ no. (%)	Other *spa* types,§ no. (%)			
FY01	2	0	2 (100)	0	0	0	0
FY02	13	4 (31)	5 (38)	4 (31)	3 (23)	3 (23)	2 (17)
FY03	21	15 (71)	6 (29)	0	15 (71)	15 (71)	15 (71)
FY04	94	77 (82)	14 (15)	3 (3)	74 (80)	70 (75)	68 (74)
FY05	166	147 (89)	15 (9)	4 (2)	154 (93)	147 (89)	138 (84)
Total	296	243 (82)	42 (14)	11 (4)	246 (83)	235 (79)	223 (76)

*MRSA, methicillin-resistant *Staphylococcus aureus;* PVL, Panton-Valentine leukocidin; ACME, arginine catabolic mobile element; FY, fiscal year.
†*spa* types t008, t024, t112, t622, t064, t068, t121, t1881.
‡*spa* types t002, t045, t242, t548, t539.
§*spa* types t018, t019, t084, t128, t160, t216, t937, t1887.

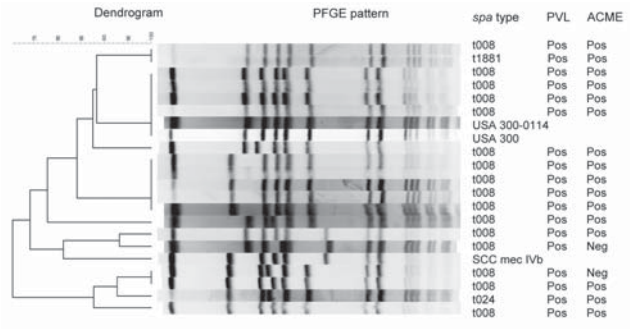

Figure 2. Pulsed-field gel electrophoresis (PFGE) of a stratified random sample of USA300 isolates and corresponding PCR results for Panton-Valentine leukocidin (PVL) and arginine catabolic mobile element (ACME). The Centers for Disease Control and Prevention's PFGE results for USA300, USA300-0114, and SCC*mec* IVb were added as controls.

were negative for PVL and ACME and similar to USA500 according to PFGE; these isolates were excluded from our definition of USA300.

Because most of these MRSA infections were SSTIs and because our ECS physicians thought they were seeing more abscesses, we looked at the rate of SSTIs in the ECS. The rate of ECS visits for SSTI significantly increased from 20 per 1,000 ECS visits in FY01 to 61 per 1,000 visits in FY05 (Figure 3). Because of concerns that some of the same patients had multiple visits, we looked at patients with SSTIs in each year. The results were similar to SSTI visits and showed an increase over the years in the number of patients with SSTIs (Table 2). The absolute number and the proportion of patients for whom culture was performed increased from FY01–03 through FY04–05 ($p<0001$, χ^2 test). We then examined the absolute number and the proportion of patients for whom cultures were performed and grew *S. aureus* (MRSA or MSSA). For our comparison of MRSA and MSSA, we chose this measurement to account for the increase in culturing. For MRSA, the absolute number and proportion of patients for whom cultures were performed and grew MRSA increased significantly from FY01–03 through FY04–05 ($p<0001$, χ^2 test). For MSSA, the absolute number of patients for whom cultures were performed and grew MSSA increased from FY01–03 through FY04–05, but the proportion of patients for whom cultures were performed and grew MSSA remained the same ($p = 0.10$, χ^2 test). Because of concerns that there may have been shifts in where care was delivered within our healthcare system, we also examined the number and rate of SSTIs for the entire VAMHCS. Absolute numbers of visits increased, with 2,020 visits in FY01, 1,972 in FY02, 2,190 in FY03, 3,337 in FY04, and 3,522 in FY05. The rate of SSTI visits also increased (2.75 SSTI visits per 1,000 visits in FY01–03 vs. 3.89 SSTI visits per 1,000 visits in FY04–05; $p<0.001$).

Discussion

During our 5-year study, we had an ≈4-fold increase in the incidence of MRSA infections, primarily SSTIs in people who had no risk factors for acquiring the infection from a healthcare setting. We showed that this increase was due to the USA300 clone and associated with an overall increase in SSTIs in our ECS and in the healthcare system as a whole. For these patients with SSTIs, the absolute number and proportion of those for whom a culture was performed and grew MRSA increased. We believe this reflects the increase in MRSA infections due to USA300. The absolute number of patients for whom a culture was performed and grew MSSA also increased, but the proportion remained the same. We believe the absolute numbers increased because more cultures were performed, not because MSSA infections increased.

Other reports of community-onset MRSA infections throughout the United States have been published (*5*,*8*,*17*–*19*). A recent publication by King et al. showed that USA300 was the predominant cause of SSTIs in the community (*8*). Carlton et al. also documented an increase in the number of total MRSA infections in San Francisco during 1996–2002 (*9*). This increase was shown to coincide with a statistically significant temporal increase in the number of community-onset MRSA infections. This study and our study support the hypothesis that CA-MRSA strains have factors that facilitate their spread in the community (*18*,*20*,*21*).

Despite the retrospective nature of our investigation, we were able to obtain 90% of the MRSA isolates for molecular typing. Molecular characterization of new cases of MRSA showed a dramatic increase in isolates with the MBQBLO repeat motif in the later years. This increase in these related *spa* types is consistent with the increase in the PFGE type USA300 seen by others (*6*,*19*). Not surprisingly, with the

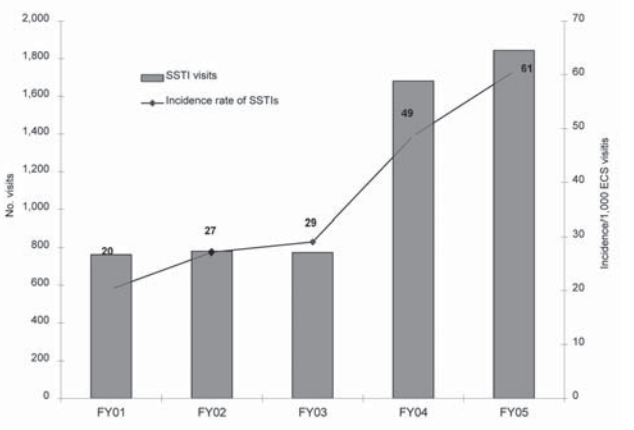

Figure 3. Visits for skin and soft tissue infections (SSTIs) in Baltimore Veterans Affairs Medical Center Emergency Care Service (ECS), 2001–2005. FY, fiscal year. FY01–03 versus FY04–05, χ^2 test, $p<0.001$.

Table 2. Microbiologic characteristics of samples taken from patients with SSTIs, Baltimore Veterans Affairs Medical Center Emergency Care Service, 2001–2005*

Year	Had SSTI, no.	SSTI was cultured, no. (%)	SSTI was cultured and grew MRSA, no. (%)	SSTI was cultured and grew MSSA, no. (%)
FY01	496	98 (20)	4 (4)	10 (10)
FY02	574	120 (21)	10 (8)	12(10)
FY03	567	99 (17)	11 (11)	7 (7)
FY04	981	292 (30)	96 (33)	37 (13)
FY05	1,070	410 (38)	172 (42)	52 (13)

*SSTIs, skin and soft tissue infections; MRSA, methicillin-resistant *Staphylococcus aureus;* MSSA, methicillin-susceptible *S. aureus.*

increase in the MBQBLO repeat motif, we also observed an increase in the virulence factors PVL and ACME. We did find 4 isolates of the *spa* type 65 that were negative for PVL and ACME and were similar to USA500 by PFGE and 5 isolates that were *spa* type 8 and positive for PVL but negative for ACME. This finding was not surprising because this phenomenon has been recently described; only isolates with the SCC *mec* IVa harbored the ACME gene (*22*).

We noted that SSTIs more than doubled during the 5 years of our investigation. The increase in SSTIs has also been observed throughout the United States. For example, a study by CDC showed that the visit rate for SSTIs during 2001–2003 was 410.7 per 10,000 persons (*23*). Although an overall increase in SSTIs was not seen, SSTIs in the ECS increased by 59% and for hospital outpatient department visits increased by 31%. These increases could be associated with the emergence of CA-MRSA infections and are consistent with our study findings, which showed that this increase was due to the USA300 clone and also with an overall increase in SSTIs.

The potential limitations of this study include the study population and its retrospective nature. Because the study population consisted of veterans who received treatment through the VA Maryland Healthcare System, and thus were mainly male patients of low socioeconomic status, the study was not a true population-based study. Although we focused only on the veteran population, we believe that our findings are consistent with those of other scientific studies and are relevant to most emergency department populations. The use of a veteran population is also an advantage. We were able to obtain more comprehensive medical information from the VA's computerized medical record system than would likely be available for a nonveteran population. This study was a retrospective review of information obtained for the clinical treatment of infections, and therefore many SSTIs were not cultured. Although the increased frequency of culturing may have led to some increase in MRSA, the fact that the proportion of SSTIs that were MSSA stayed the same suggests that the increase in MRSA infections is real.

In conclusion, we showed an increase in CA-MRSA infections of the USA300 clone and an increase in SSTIs during a 5-year period in the ECS and systemwide at the VAMHCS. The emergence of the USA300 clone has led to not only to an increase in CA-MRSA infections but also an overall increase in SSTIs in our patient population. This finding suggests that this clone is becoming more virulent with a greater propensity to cause SSTIs.

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Dr Johnson is assistant professor at the University of Maryland School of Medicine, Department of Pathology, and associate director of the clinical microbiology laboratory at the University of Maryland Medical Center. Her main research interest is antibiotic resistance, focusing on MRSA and resistant mobile genetic elements in gram-negative organisms, including extended-spectrum β-lactamases and plasmid-mediated AmpC β-lactamases.

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Classic Scrapie in Sheep with the ARR/ARR Prion Genotype in Germany and France

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In the past, natural scrapie and bovine spongiform encephalopathy (BSE) infections have essentially not been diagnosed in sheep homozygous for the $A^{136}R^{154}R^{171}$ haplotype of the prion protein. This genotype was therefore assumed to confer resistance to BSE and classic scrapie under natural exposure conditions. Hence, to exclude prions from the human food chain, massive breeding efforts have been undertaken in the European Union to amplify this gene. We report the identification of 2 natural scrapie cases in ARR/ARR sheep that have biochemical and transmission characteristics similar to cases of classic scrapie, although the abnormally folded prion protein (PrP^{Sc}) was associated with a lower proteinase-K resistance. PrP^{Sc} was clearly distinct from BSE prions passaged in sheep and from atypical scrapie prions. These findings strongly support the idea that scrapie prions are a mosaic of agents, which harbor different biologic properties, rather than a unique entity.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases in sheep and goats (scrapie), cattle (bovine spongiform encephalopathy [BSE]), and humans (Creutzfeldt-Jakob disease [CJD]). A variant form of CJD (*1*) was discovered in 1996 and was linked to the BSE epidemic in the United Kingdom and elsewhere. Classic scrapie is caused by a variety of prion strains that can be distinguished from one another by their biologic and biochemical features (*2*). Recently, so-called atypical scrapie strains that have remarkably different biochemical and transmission characteristics have been discovered (*3*,*4*). Although the transmissibility of a particular sheep scrapie isolate to nonhuman primates has been demonstrated (*5*), no epidemiologic data have linked scrapie in small ruminants to human CJD cases (*6*). TSE susceptibility in sheep is controlled mainly by polymorphisms in the monocistronic *PRNP* gene that encodes for normal cellular protein (PrP^{C}). Three major mutations are associated with sheep susceptibility or resistance to classic scrapie and BSE: at codons 136 (A or V), 154 (R or H), and 171 (R, Q, or H) (*7*). Animals with genotypes $V^{136}R^{154}Q^{171}$/VRQ, ARQ/VRQ, ARQ/ARQ , and VRQ/ARH PrP are most susceptible to scrapie (*8*). In the past 20 years, no TSE cases have been found in ARR/ARR sheep in Europe, although thousands of scrapie-diseased animals have been genotyped. However, 1 report, albeit heavily questioned, has been made in the literature of a possible case in an ARR/ARR sheep in Japan (*9*). Therefore, this genotype was considered to confer full resistance to BSE and scrapie (*7*) (for a full review see [*10*]). To minimize the risk of humans acquiring TSE by consuming animal products, massive breeding programs involving PrP-genotyping of millions of sheep were initiated in the European Union (EU).

However, the successful transmission of BSE prions to ARR/ARR sheep showed that the resistance of this genotype toward the TSE agent was not absolute (*11*). Recently, the identification of previously unrecognized so-called atypical scrapie in sheep of various genotypes, including ARR/ARR, has reinforced this statement (*4*). We report here the identification and characterization of 2 natural classic scrapie cases in sheep of the ARR/ARR genotype, which are clearly different from BSE and atypical scrapie.

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Methods

ELISA, Scrapie-associated Fibril, and Conventional Immunoblots

For ELISA detection of PrP^{Sc}, commercial TSE rapid tests, TeSeE Sheep/Goat (Bio-Rad, Marnes-la-Coquette, France), were used according to the manufacturer's recommendations. Scrapie-associated fibrils from the brain stem of infected ovines were purified and immunoblotted, according to the protocol by the World Organization for Animal Health (*12*). In this assay, we used the monoclonal antibody L42, which binds the 145–150 sequence of PrP(YEDRYY). Visualization was achieved by using the chemiluminescence substrate CDP-Star (Tropix, Bedford, MA, USA) and the Bio-Rad VersaDoc imaging system, and signals were analyzed by using the Quantity One quantification software (Bio-Rad).

The TeSeE Western Blot Kit (Bio-Rad) was used according to the manufacturer's recommendations. This kit uses the monoclonal antibody SHa-31, which binds the 145–152 sequence of PrP (YEDRYYRE).

Proteinase K (PK) Resistance Assay

Ten percent of brain homogenates from the brain of a 5-year-old sheep with scrapie in France, designated S83, were analyzed by the TeSeE Sheep/Goat ELISA as recommended by the manufacturer. Each sample was diluted in PrP^{Sc}-negative ARR/ARR sheep 10% brain homogenate until an optical density (OD) of 1.5 to 1.7 was obtained in the ELISA. Equilibrated homogenate aliquots were submitted to PK digestion with a concentration ranging from 50 to 500 μg/mg. PrP^{Sc} was precipitated (as in the basic TeSeE Sheep/Goat ELISA) and the pellet dissolved in 25 μL buffer C1, incubated for 5 min at 100°C, and diluted 12-fold in R6 reagent. Samples were run in triplicate and detected by using the TeSeE Sheep/Goat ELISA.

Bioassay

Twenty microliters of 10% brain homogenates were intracerebrally inoculated into C57Bl6, RIII, VM, and Tg-shp XI mice that overexpress the ovine PrP^{ARQ}, into Tg338 mice that overexpress the ovine PrP^{VRQ}, and into Tgbov XV mice that overexpress the bovine PrP. Incubation times were recorded, and tissue samples from clinically affected mice were collected and preserved.

Lesion Profiling and Paraffin-Embedded Tissue (PET) Blot

Lesion profiles were established by following the standard method of Fraser and Dickinson (*13*) and using 6 brains per isolate. For the PET blots, we used sections from positive transgenic mice (*14*).

PRNP Sequencing

DNA was extracted from brain tissue of the scrapie-positive sheep with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). PCR-amplification and sequencing of a 970-bp PCR fragment, including the complete coding region of the *PRNP,* were done as described previously (*15*). Additionally, the PCR fragment was cloned with the pGEM-T Easy Vector System (Promega GmbH, Mannheim, Germany). Plasmid DNA of 12 recombinant clones was sequenced, and these sequences were compared with GenBank *PRNP* sequence U67922.

Results

Histories of 2 ARR Homozygous Sheep with Scrapie

The case in an ARR homozygous sheep in Germany was initially diagnosed during 2004 in the course of the intensified EU-wide testing of fallen stock and slaughter animals. A recent retrospective genotyping study on all 230 small ruminant TSE cases diagnosed in Germany between 2000 and 2006 led to the identification of this ARR/ARR case.

This animal showed borderline reactivity in the Bio-Rad TeSeE rapid test on the brain stem (initial result, OD 0.279; cut-off 0.217; test repetition in duplicate OD 0.259/0.256; cut-off 0.219) and was positive in the Western blot confirmation test (Figure 1). The animal (S115/04) was a 2- to 4-year-old ewe of the black-headed German mutton breed. Apart from a general loss of condition, the animal did not show any clinical signs suggestive of scrapie. After the official confirmation of the case, all animals in the herd of origin (n = 1,297) were genotyped regarding PrP codons 136, 154, and 171. The frequencies of PrP genotypes in sheep >1 year of age (n = 871) were 46.7% (ARR/ARQ), 35.4% (ARR/ARR), 16.9% (ARQ/ARQ), 0.7% (ARR/VRQ), and 0.1% for each of the genotypes ARQ/VRQ, ARR/AHQ, and AHQ/ARQ. In accordance with the EU regulations, all sheep not carrying at least 1 ARR haplotype were slaughtered, and rapid tests for TSE were conducted. No further TSE case was detected among these sheep. The origin of the infection in this outbreak remains unknown, but rams originating from another flock in Germany with classic scrapie were apparently used in this flock in 1999.

We also examined brain, tonsil, or retropharyngeal lymph node samples from >1,700 sheep with clinically suspected scrapie, collected in France from 1993 through 2001, and identified a single case in a sheep with the ARR/ARR genotype. This animal, S83, was a 5-year-old sheep born in 1995 that had some clinical symptoms of scrapie but for which no detailed symptoms are described. The brain stem sample of S83 was positive by ELISA in the Bio-Rad TeSeE Sheep/Goat rapid test (OD 2.4; cut-off 0.212) and gave a clearly positive signal on Western blot (Figure 1).

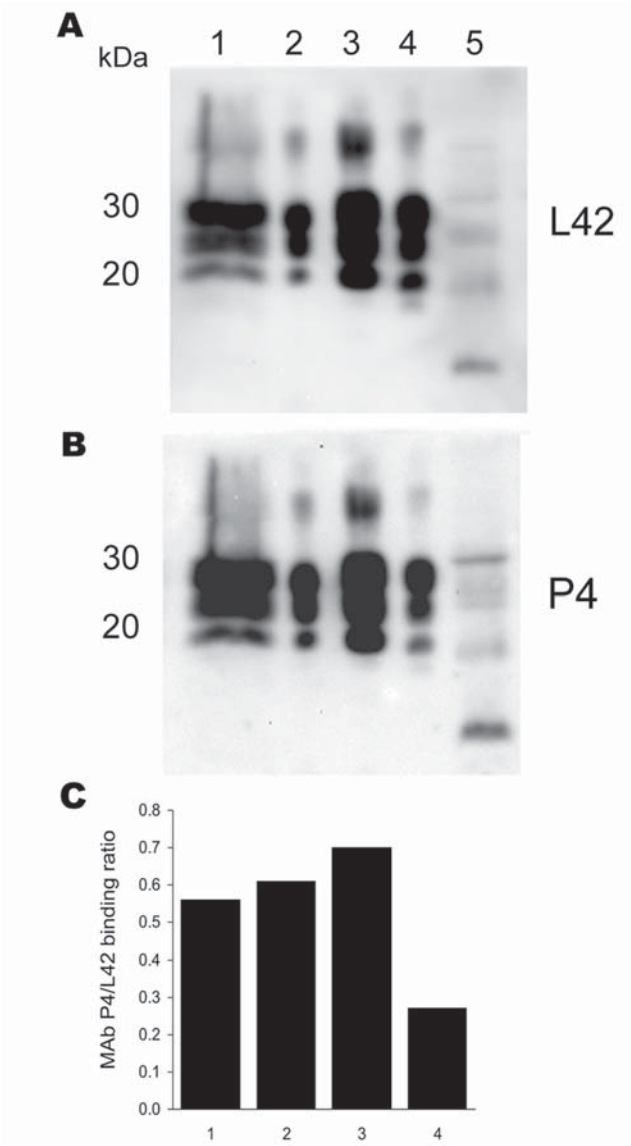

Figure 1. Antibody-binding patterns of the prion protein (PrP^{Sc}) associated with cases of ARR/ARR scrapie in France and Germany. A) and B) Western blots showing the differences in monoclonal antibody (MAb) P4 binding compared with the internal standard MAb L42 of PrP^{Sc} derived from S115/04 (ARR/ARR Germany), S83 (ARR/ARR France), ovine ARQ/ARQ bovine spongiform encephalopathy (BSE), and S95 (classic scrapie) cases. Banding intensities were quantified by photoimaging, and binding ratios were calculated. Note the significantly weaker P4 binding to the ovine BSE sample. Lane 1, S115/04; lane 2, S83; lane 3, S95; lane 4, ovine BSE; lane 5, atypical S15. C) Relative MAb binding ratios for lane nos. 1–4 in the Western blots shown in A) and B).

Tonsil and retropharyngeal lymph nodes from this animal were negative for scrapie by ELISA and Western blot. In addition, conventional histologic examination found a severe *Listeria* infection, but no vacuoles, in the brain stem. No detailed information was available on the flock of origin and the genetic structure of that flock. No further scrapie cases were reported until 2001; the farm was then closed.

Genetic Analysis

In addition to the routine genotyping at codons 136–154 and 171, *PRNP* gene sequences from the 2 scrapie cases were determined by sequencing of PCR amplificates from brain-derived DNA to compensate for the possibility of a genetic chimerism in blood. For both scrapie cases S115/04 and S83, *PRNP* sequence analysis unanimously showed the homozygous *PRNP* genotype ARR/ARR. There were no differences between the *PRNP* sequences determined by direct sequencing of the PCR products and by sequencing of 12 cloned PCR fragments for each case, which excludes the possibility that *PRNP* haplotypes other than ARR were present in the analyzed tissue.

Biochemical Properties of PrP^{Sc}

The Western blot of S115/04 and S83 showed the 3-banded PrP^{Sc} pattern that is typically associated with scrapie. The molecular masses of the nonglycosylated PrP^{Sc} bands were ≈21 kDa, and both animals lacked the 12-kDa PrP^{Sc} band seen in atypical scrapie (Figure 1). Additionally, the nonglycosylated PK-treated PrP^{Sc} bands of S115/04 and S83 showed lower electrophoretic mobility than PrP^{Sc} derived from BSE-infected sheep (ARQ/ARQ). The monoclonal antibody P4, which recognizes PrP^{Sc} derived from scrapie-affected sheep, but has a lower affinity to BSE PrP^{Sc}, clearly detected both S115/04- and S83-derived PrP^{Sc} and, to a substantially lesser extent, PrP^{Sc} from a BSE-infected sheep. These results strongly support the contention that both isolates differ from those that cause BSE and atypical scrapie (Figure 1; Figure 2, panel A).

The limited amount of sample material available from case S115/04 prevented any further biochemical characterization. For case S83, however, it was possible to compare the PK resistance of PrP^{Sc} with that of 20 randomly selected classic scrapie cases, 1 case of BSE in an ARR/ARR sheep, and 1 atypical case in an ARR/ARR sheep. S83 PrP^{Sc} was found to have a significantly lower level of PK resistance than that found in classic scrapie PrP^{Sc}. However, resistance was similar to that of PrP^{Sc} in experimentally BSE-infected ARR/ARR sheep and PrP^{Sc} levels of animals with atypical scrapie (Figure 2, panel B).

Bioassay in Transgenic Mice

Samples from both infected sheep were inoculated into a panel of conventional (C57Bl6, RIII, and VM) mice, transgenic bovinized (Tgbov XV) mice, and ovinized (ARQ Tgshp XI [unpub. data], VRQ Tg338) mice. These transmission experiments are ongoing. However, for the isolate S83 from France, results in transgenic ovinized VRQ mice (Tg338) are available.

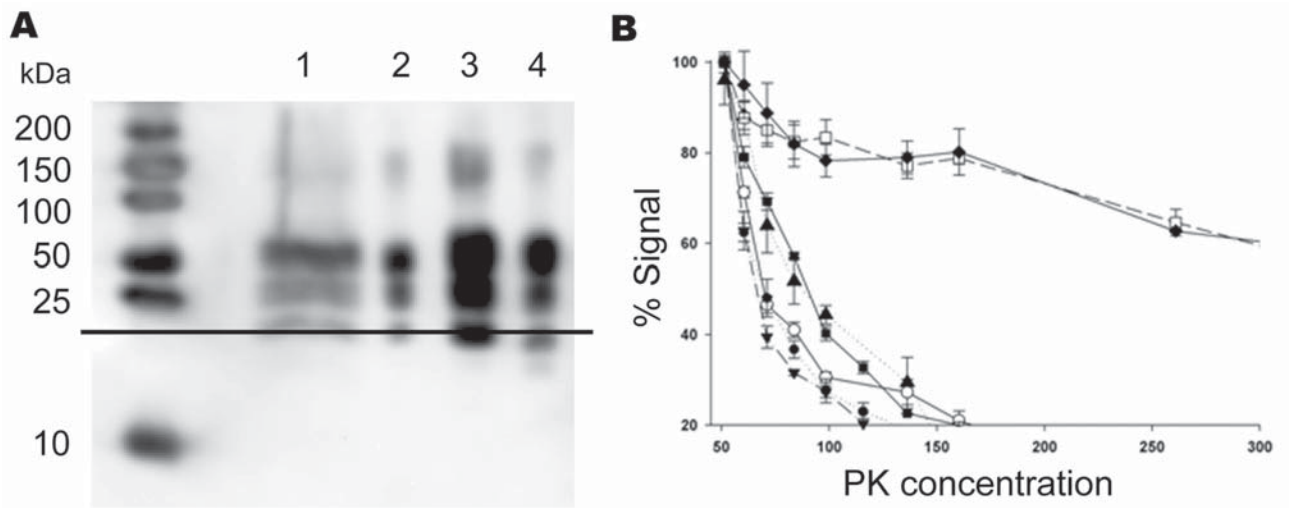

Figure 2. Biochemical characterization of the prion protein (PrP^{Sc}) associated with ARR/ARR cases in France and Germany. A) Western blot (stained by monoclonal antibody L42) illustrating that protein kinase (PK)– treated ovine bovine spongiform encephalopathy (BSE) PrP^{Sc} has ≈1-kDa lower molecular mass than PrP^{Sc} from the scrapie cases. Lane 1, S115/04, molecular mass (MM) 20.95 kDa; lane 2, S83, MM 19.96 kDa; lane 3, S95 classic scrapie, MM 19.64 kDa; lane 4, ovine BSE, MM 18.85 kDa. B) PrP^{Sc} PK sensitivity measured by using brain from S83 scrapie case in France (▲), ARR/ARR BSE in sheep (○), ARQ/ARQ BSE in sheep (●), BSE from bovines (■), an ARR/ARR atypical scrapie case (▼), and 20 randomly selected isolates from sheep with scrapie in France (2 cases shown, represented as and ◆). PrP^{Sc} ELISA measurements were performed by using the TeSeE Sheep/Goat rapid test (Bio-Rad) after brain homogenate digestion using a PK concentration ranging from 50 µg/mL to 500 µg/mL. Three tests were performed for each sample and PK concentration.

In intracerebrally inoculated Tg338 mice (20 µL of a 10% brain homogenate), nervous symptoms consistent with TSE developed after a mean incubation period of 309 ± 35 days. In all mice, PrP^{Sc} was detected by using Western blot. For Tg 338 mice, the mean incubation period was 239 ± 32 days for the atypical scrapie isolate, and the incubation time for BSE prion previously passaged through ARQ/ARQ sheep was 534 ± 25 days.

Both lesion profile and PET blot PrP^{Sc} distribution in Tg 338 mice enabled a clear differentiation between the S83 isolate, atypical scrapie, and BSE in sheep (Figure 3). The lesion profile observed in Tg338 (Figure 3, panel A) inoculated with atypical scrapie was similar to that described in published data for 11 atypical scrapie cases in France (*16*).

S83-isolate–infected Tg338 mice PrP^{Sc} reproduced a Western blot signature similar to that of the initial cases (electromobility and glycotype) and was distinct from that of PrP^{Sc} purified from Tg338 mice infected with either BSE in sheep or atypical scrapie (Figure 4, panel A). After passage of the 1) S83 isolate, 2) isolates from the atypical scrapie case and BSE in sheep, and 3) 3 independent classic scrapie isolates passaged in Tg338 mice, the PK resistance of the PrP^{Sc} was measured (Figure 4, panel B). Because Tg338 mice overexpress the VRQ sheep allele and, consequently, PrP^{Sc} produced in this mouse model derives from the conversion of VRQ-PrP^{C}, the finding that PrP^{Sc} PK resistance in these mice was similar to that of the original isolate was surprising. Thus, the lower PrP^{Sc} PK resistance observed in S83 isolate seems not to depend on the *PRNP* sheep genotype but is rather an intrinsic property of the scrapie strain involved.

Discussion

Epidemiologic and genetic data collected in recent decades have indicated that sheep carrying the PrP^{C}-encoding ARR haplotype only are resistant to natural TSE infections, whereas those carrying the homozygous VRQ or ARQ haplotypes are highly susceptible. This assumption has been supported by genotyping data for thousands of sheep with scrapie worldwide: only 1 homozygous ARR carrier has been found. A case of classic scrapie in an ARR/ARR sheep in Japan was reported more than a decade ago (*7*). However, the validity of this diagnosis has been heavily challenged when it could not be reconfirmed independently, because no suitable frozen or formalin-fixed material was available. Our current report shows that classic scrapie cases can occur in homozygous ARR sheep and suggests that even the former report may have been an imperfect first demonstration of such a case.

The apparent resistance of sheep of the ARR/ARR genotype to both natural scrapie and experimental BSE has triggered national authorities since the late 1990s (e.g., United Kingdom, the Netherlands, France), and since 2001 the EU, to implement a genetic selection and culling policy in sheep to protect the human food chain from small ruminant TSEs and to eradicate TSE from affected flocks.

This global approach, even if valuable, considered scrapie as a single entity and did not take into account any

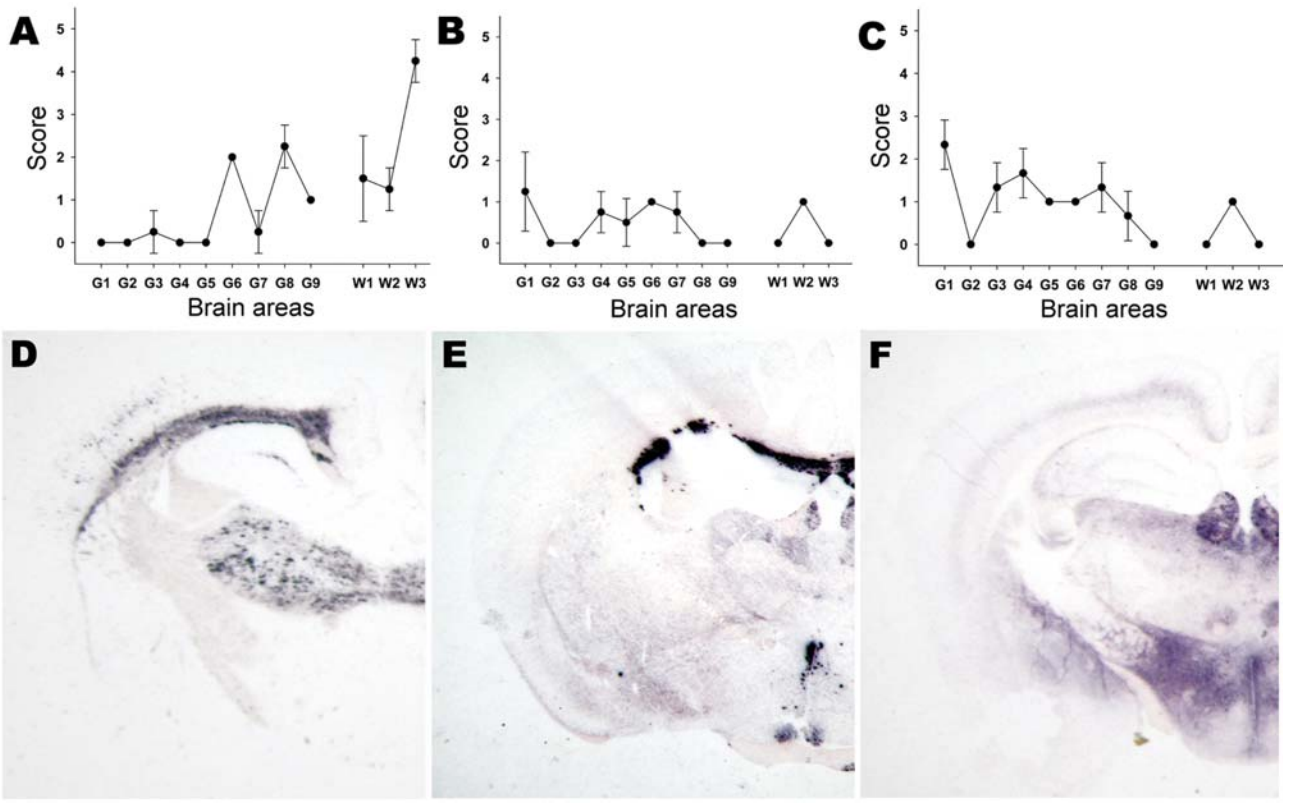

Figure 3. Lesion profiling (A, B, C) and paraffin-embedded tissue blot characterization of prion protein (PrP^{Sc}) deposition at thalamic level (D, E, F). Tests were performed by using formalin-fixed brain from Tg338 mice (expressing the VRQ PrP ovine variant) inoculated with (A, D) ARR/ARR atypical case (B, E) bovine spongiform encephalopathy (BSE) brain from an ARR/ARR sheep (intracerebral inoculation), and (C, F) case S83. Each lesion profile was carried out by using 6 animals. Detection of PrP^{Sc} was achieved by using the monoclonal antibody Sha31.

kind of TSE agent biodiversity. Sheep TSE agents have strikingly different abilities to replicate in hosts expressing a spectrum of PrP variants. Sheep with genotype ARQ/ARQ in scrapie flocks are commonly affected by the disease (*8*). However, a historical sheep scrapie brain pool (SSBP-1) transmits easily to VRQ homozygous or heterozygous sheep but not to ARQ/ARQ animals (*17*). Similarly atypical scrapie cases (including the "Nor98" type) are more frequently found in AHQ and $AF_{141}RQ$ haplotype carriers than in sheep carrying exclusively other haplotypes. However, atypical cases have also been found in ARR/ARR animals (*18*,*19*). Similarly, the ability of BSE to develop in ARR/ARR sheep was observed after experimental parenteral inoculation. However, in this case, higher transmission rates and shorter incubation periods were observed in sheep of the other genotypes, such as ARQ/ARQ, and AHQ/AHQ (*20*,*21*). The susceptibility of ARR/ARR sheep to an oral BSE challenge was reported most recently (*22*).

Both BSE and atypical scrapie PrP^{Sc} have a characteristic molecular signature, which allows rapid and reliable biochemical discrimination from each other and from classic scrapie PrP^{Sc} (*3*,*23*). In both cases of scrapie in ARR/ARR sheep in France and Germany, abnormal PrP^{Sc} harbored features (apparent molecular mass and glycotype) that were similar to those observed in classic scrapie. However, at least in the S83 case, PrP^{Sc} seemed to have a remarkably lower PK resistance than that observed in a panel of scrapie isolates. This observation sustains the idea that the involved agent could belong to a particular scrapie agent group that cannot be directly identified by using the current biochemical criteria for TSE agent discrimination.

The successful propagation of the S83 isolate in Tg338 mice that express the ovine VRQ haplotype and the persistence of its original biochemical signature (including the low PK resistance) allow the inference that this scrapie agent could also be present and could naturally propagate in sheep that harbor genotypes other than ARR/ARR. The transmissibility and contagiousness of the S83 isolate are currently under investigation in experimentally challenged sheep. These experiments should produce a better understanding of the susceptibility of each genotype to this agent and its capacity to spread efficiently in sheep flocks.

The discovery of these 2 cases clearly indicates that the genetic resistance of ARR/ARR sheep to the so-called clas-

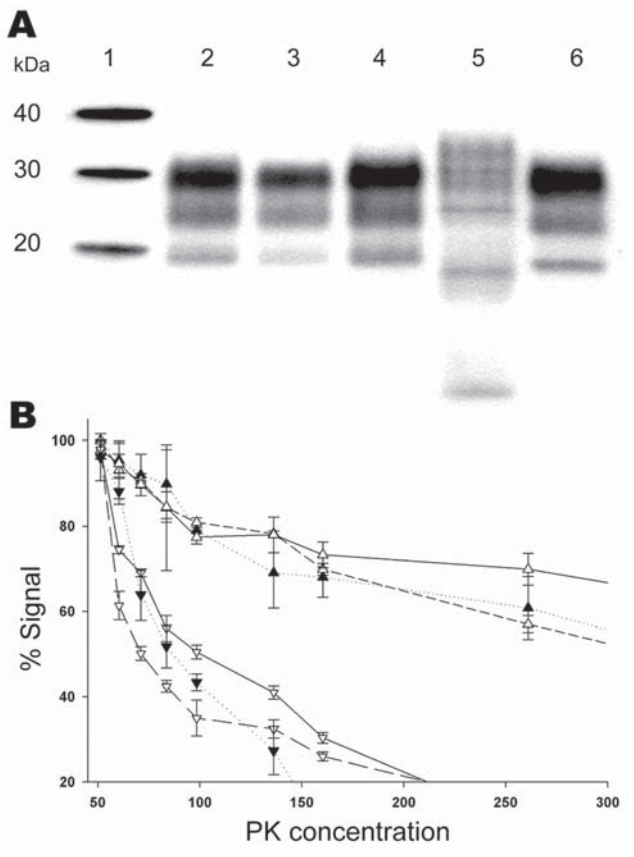

Figure 4. Biochemical properties of prion protein (PrP^{Sc}) associated with the ARR/ARR scrapie case S83 from France after passage in Tg 338 VRQ mice. A) Western blot mobility of the original S83 ARR/ARR case (lane 3) and S83 passaged in Tg338 (lane 4) were similar and comparable to a classic scrapie isolate (Langlade, lane 2). PrP^{Sc} WB profile of ARR/ARR bovine spongiform encephalopathy (BSE) in sheep (lane 6) and profiles of atypical scrapie case isolates (lane 5) passaged into Tg338 mice were readily distinguishable by their banding pattern or electromobility. B) PrP^{sc} protein kinase (PK) sensitivity of the original S83 isolate (▼) and a classic scrapie isolate (Langlade) (△) compared with S83 (▽) and Langlade (▲) that had been passaged in Tg338 (2 different mice for each isolate). Triplicate ELISA measurements were performed by using the TeSeE Sheep/Goat rapid test (Bio-Rad), after brain homogenate digestion with PK concentration ranging from 50 µg/mL to 500 µg/mL.

sic scrapie agent is not absolute. It also provides evidence that, rather than being a single entity, scrapie is a mosaic of infectious agents harboring different biologic properties in its natural host. Finally, although many thousands of cases of classic scrapie have been reported in sheep of other PrP genotypes and hundreds of thousands of rapid tests have been performed in Europe since the implementation of active TSE surveillance in small ruminants began in 2001, the discovery of these 2 ARR/ARR cases supports the idea that such infections are extremely rare.

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ANOTHER DIMENSION

The Calf-Path

–Sam Walter Foss, Public Domain

One day through the primeval wood
A calf walked home as good calves should;
But made a trail all bent askew,
A crooked trail as all calves do.
Since then three hundred years have fled,
And I infer the calf is dead.
But still he left behind his trail,
And thereby hangs my moral tale.
The trail was taken up next day
By a lone dog that passed that way;
And then a wise bell–wether sheep
Pursued the trail o'er vale and steep,
And drew the flock behind him, too,
As good bell–wethers always do.
And from that day, o'er hill and glade,
Through those old woods a path was made.
And many men wound in and out,
And dodged and turned and bent about,
And uttered words of righteous wrath
Because 'twas such a crooked path;
But still they followed – do not laugh -
The first migrations of that calf,
And though this winding wood-way stalked
Because he wobbled when he walked.
This forest path became a lane
That bent and turned and turned again;
This crooked lane became a road,
Where many a poor horse with his load
Toiled on beneath the burning sun,
And thus a century and a half
They trod the footsteps of that calf.
The years passed on in swiftness fleet,
The road became a village street;
And this, before men were aware,
A city's crowded thoroughfare.
And soon the central street was this
Of a renowned metropolis;
And men two centuries and a half
Trod in the footsteps of that calf.
Each day a hundred thousand rout
Followed this zigzag calf about
And o'er his crooked journey went
The traffic of a continent.
A hundred thousand men were led
By one calf near three centuries dead.
They followed still his crooked way.
And lost one hundred years a day,
For thus such reverence is lent
To well-established precedent.
A moral lesson this might teach
Were I ordained and called to preach;
For men are prone to go it blind
Along the calf-paths of the mind,
And work away from sun to sun
To do what other men have done.
They follow in the beaten track,
And out and in, and forth and back,
And still their devious course pursue,
To keep the path that others do.
They keep the path a sacred groove,
Along which all their lives they move;
But how the wise old wood-gods laugh,
Who saw the first primeval calf.
Ah, many things this tale might teach —
But I am not ordained to preach.

Babesia sp. EU1 from Roe Deer and Transmission within *Ixodes ricinus*

Sarah Bonnet,* Maggy Jouglin,* Monique L'Hostis,† and Alain Chauvin†

We report in vitro culture of zoonotic *Babesia* sp. EU1 from blood samples of roe deer in France. This study provides evidence of transovarial and transstadial transmission of the parasite within *Ixodes ricinus*, which suggests that this tick could be a vector and reservoir of EU1.

Babesiosis is a zoonosis caused by intraerythrocytic piroplasms of the genus *Babesia*, which are transmitted by ticks (*1*). In Europe, ≈30 human cases of babesiosis have been reported over the past 50 years and have been traditionally attributed to infection with the bovine parasite *B. divergens* transmitted by *Ixodes ricinus* (*2*,*3*). However, in 2003, Herwaldt et al. described the first molecular characterization of a new *Babesia* species, *Babesia* sp. EU1, isolated from 2 persons in Austria and Italy (*4*). Since this description, EU1 has been recovered from roe deer in Slovenia (*5*) and from *I. ricinus* in Slovenia (*6*) and Switzerland (*7*,*8*).

Babesia species EU1 merits increased attention as a potential agent of emerging tickborne disease in humans because its suspected vector, *I. ricinus*, is the most prevalent and widely distributed tick in Europe and frequently bites humans. To evaluate the public health importance of EU1, its vector, animal reservoir hosts, and geographic distribution must be identified. We identified EU1 in roe deer and in *I. ricinus* in western France.

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The Study

In January 2005 and January 2006, 89 roe deer at the Wild Fauna Reserve of Chizé (Deux Sèvres, France) were captured; blood was obtained through the jugular vein and analyzed for infection by *Babesia* spp. Parasites were isolated from autologous roe deer erythrocytes as described (*9*), except that 20% fetal calf serum (FCS) was used. Cultures were monitored for parasitemia by examination of Giemsa-stained thin blood smears. Parasites were then adapted to culture in blood from deer (*Dama dama)* from the Jardin des Plantes of Nantes (Loire Atlantique, France) and from sheep reared in our laboratory. Adaptation was conducted as described (*10*), except that 20% FCS was also used.

A total of 150 μL parasite genomic DNA was then prepared from 10-mL cultures (10% parasitemia, 2.5% hematocrit) according to the protocol of a commercial extraction kit (Promega, Madison, WI, USA) on merozoite preparations obtained by Percoll gradient centrifugation (Amersham, Uppsala, Sweden) with a density of 1.08 g/mL in 0.15 M NaCl. PCR amplifications were performed with 10 μL DNA and BAB primers (Table 1) as described (*11*). For positive samples, 5 μL DNA was subjected to a second amplification with EU1 primers (Table 1) as described by Hilpertshauser et al. (*8*), except that the annealing temperature was 63°C and uracil DNA glycosylase was not used.

During January 2005, 31 of 79 roe deer analyzed were infected with *Babesia* spp., as shown by parasite culture and PCR amplification with BAB primers. Of 29 cultures tested for EU1 with the corresponding primers, 59% were positive, which indicated an estimated global EU1 prevalence of 23% (Table 2). In January 2006, 5 of 10 cultures tested contained *Babesia* spp. parasites. Sequencing of the complete 18S rRNA gene from subcultures in autologous deer and sheep erythrocytes amplified with the primer set CRYPTO (Table 1) (*4*) showed that 2 of these cultures (C210 and C201) had 100% identity with the EU1 human strain (GenBank accession no. AY046575) (*4*). The unique sequence obtained has been deposited in GenBank (accession no. EF185818).

Table 1. Nucleotide sequences of PCR primers used for amplification and sequencing of 18S rRNA genes of *Babesia* spp.*

Primer	Specificity	Sequence (5′→3′)	Annealing temperature, °C	Fragment size, bp	Reference
BAB	*Babesia*/*Theileria* spp.		60	359	(*11*)
GF2		GYYTTGTAATTGGAATGATGG			
GR2		CCAAAGACTTTGATTTCTCTC			
EU1	*Babesia* sp. EU1		63	362	(*8*)
Up		GTTTCTGMCCCATCAGCTTGAC			
Down		AGACAAGAGTCAATAACTCGATAAC			
CRYPTO	Apicomplexa		65	1,727	(*4*)
F		AACCTGGTTGATCCTGCCAGTAGTCAT			
R		GAATGATCCTTCCGCAGGTTCACCTAC			

*For parasites from tick samples, no sequence could be obtained with primer set CRYPTO because such primers likely hybridize to the *Ixodes ricinus* 18S rRNA gene and preferentially amplified this gene, probably because of its relative abundance.

In January 2005, a total of 106 engorged female adult *I. ricinus* were collected from the 31 roe deer harboring *Babesia* spp. Ticks were then reared in the laboratory at 22°C and a relative humidity of 80%–90%. Forty-two ticks (from 22 roe deer) laid eggs from which larvae were analyzed for parasites with BAB primers as described (*11*); 64% of larvae samples had a positive reaction. Amplification products from egg sample E177.3 and larva sample L177.3 that were sequenced showed 100% identity with the 18S rRNA gene of EU1 (*4*). The sequence has been submitted to GenBank (accession no. EF185819). Among positive samples, 6 of 15 analyzed for EU1 with specific primers showed a positive reaction (Table 2).

Table 2. PCR detection of *Babesia* sp. EU1 in blood samples from roe deer and in *Ixodes ricinus* larvae hatched from engorged females collected from roe deer*

Roe deer Identification	Primer BAB	Primer EU1	
	Cultures	Cultures	Larvae
C105	+	+	
C107	+	+	L107.1 (+)
			L107.2 (–)
C109	+	+	L109 (+)
C110	+	+	L110.2 (–)
C112	+	+	L112.1 (–)
			L112.2 (–)
C115	+	–	
C117	+	–	
C123	+	–	
C128	+	–	L128.3(+)
C129	+	+	
C139	+	+	
C151	+	+	L151 (+)
C156	+	+	
C162	+	+	
C163	+	+	
C164	+	–	L164 (–)
C167	+	+	
C171	+	+	L171.1 (–)
C172	+	–	
C176	+	–	L176.7 (–)
C177	+	–	L177.1 (–)
			L177.2 (–)
			L177.3 (+)
C179	+	–	L179.1 (+)
C180	+	+	
C185	+	–	
C188	+	–	
C193	+	–	
C169	+	+	
C189	+	+	
C157	+	+	
Total positive (%)	29	17 (58.62)	6/15 (40)

*+, positive amplification of a product of the expected size; –, no amplification.

Conclusions

Isolation of EU1 from roe deer in France confirms that these animals are reservoir hosts of the parasite and that EU1 is not restricted to 1 geographic area in Europe. A survey conducted in Slovenia showed that 21.6% of 51 roe deer tested were infected with EU1 (*5*) and a similar prevalence (23%) was observed.

To our knowledge, this is the first isolation of EU1 in culture in homologous erythrocytes and erythrocytes from other ruminants. Until now, *Babesia* sp. EU1 has only been detected in roe deer (*5*) and humans (*4*). It has also been detected in *I. ricinus* collected from sheep and goats in Switzerland (*8*); however, the ticks in that study may have acquired the infection at a preceding stage during a blood meal taken on another host.

In a study in Slovenia in 1997, 2.2% of 135 *I. ricinus* collected by flagging vegetation were positive by PCR for EU1 (*6*,*12*). PCR studies in Switzerland that examined ticks collected from domestic and wild ruminants with unknown parasitologic status showed that 1%–2% contained EU1 DNA (*7*,*8*). In our study, 40% of larvae samples from female ticks collected on *Babesia*-infected roe deer were infected with EU1. We assume that ticks do not necessarily become infected or transmit the parasite to the next generation after a blood meal on a EU1-infected host because 5 larval pools that originated from female ticks collected on EU1-infected roe deer were not infected (Table 2).

DNA sequences of the 18S rRNA gene were identical in parasites isolated from roe deer (C201 and C210) or *I. ricinus* samples. This finding indicates that deer and ticks were infected with the same organism, which may be transmitted by the tick. In addition to *I. ricinus,* EU1 DNA has been isolated from *Haemaphysalis punctata* ticks in Switzerland (*8*). However, during this survey, entire ticks or the apical part of fully engorged females were tested. Positive results from such samples indicate infection status only, not proof of the vectorial capacity of the tick (*11*).

We report that EU1 is transmitted within *I. ricinus* and that transovarial transmission occurs in this tick, as shown by detection of parasite DNA in eggs and larvae from females collected on roe deer. Some EU1-positive eggs and larvae can originate from adults engorged on EU1-uninfected roe deer, as observed in 3 roe deer (L128.3, L177.3, and L179.1). This finding suggests that the parasite was acquired during a preceding blood meal and that transstadial transmission occurred, at least from nymph to adult. Further investigations are needed to clarify the ability of *I. ricinus* to acquire and transmit *Babesia* sp. EU1. This species, which has been isolated from 2 human cases of babesiosis, should be studied to determine other potential reservoir hosts because of its potential as an emerging zoonotic pathogen.

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Pathogenic Hantaviruses, Northeastern Argentina and Eastern Paraguay

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We describe the first, to our knowledge, cases of hantavirus pulmonary syndrome in northeastern Argentina and eastern Paraguay. Andes and Juquitiba (JUQ) viruses were characterized. JUQV was also confirmed in 5 *Oligoryzomys nigripes* reservoir species from Misiones. A novel *Akodon*-borne genetic hantavirus lineage was detected in 1 rodent from the Biologic Reserve of Limoy.

Members of the genus *Hantavirus* (family *Bunyaviridae*) are commonly transmitted to humans through rodents and may cause 2 severe human diseases: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (HPS) (*1*). The number of recognized human cases and the number of distinct hantavirus genotypes identified have increased during recent years in Argentina and in the 3 southern HPS-endemic states of Brazil (*2*,*3*). Six pathogenic Andes virus (ANDV) lineages that cause HPS have so far been found to circulate in Argentina in 3 HPS-endemic areas: Oran and Bermejo (BMJ) in the north; Lechiguanas (LEC), Hu39694, and AND Cent (Central) Plata in the central provinces; and ANDV in the South (*4*–*7*).

Different hantavirus genetic lineages associated with HPS cases were reported in Brazil, such as Juquitiba virus (JUQV), Castelo dos Sonhos virus (CASV), and Araraquara virus (ARAV) (*8*). Recently, pathogenic hantaviruses from Parana, southern Brazil, have been reported to belong to the same clade as the *Oligoryzomys nigripes*–associated strains (*9*). In western Paraguay, cases were associated with Laguna Negra virus (LNV) from *Calomys laucha* (*10*). In northern Argentina and Bolivia, LNV was obtained from *C. callosus* (*11*,*12*). No cases have been reported in other areas of Paraguay, to our knowledge, until this study.

We describe 3 HPS cases that occurred in northeastern Argentina, Misiones Province, which borders 2 other hantavirus-endemic countries, Brazil and Paraguay. We also describe what we believe to be the first case that occurred in eastern Paraguay and analyzed rodents captured in Misiones and characterized a novel genetic hantavirus lineage from the Biologic Reserve of Limoy in eastern Paraguay.

The Study

Three HPS cases were confirmed in Misiones Province, Argentina, in the following patients: a 14-year-old boy from Santa Ana in November 2003, a 28-year-old man from Leandro N. Alem in December 2003, and a 12-year-old boy living in the Dos Arroyos locality of Alem city. In January 2005, HPS was confirmed in a 19-year-old girl living in Pirapo, a rural area of Itapua Department, Paraguay. Figure 1 shows the approximate geographic location of exposure sites for patients included in this study. Clinical manifestations in the 4 patients studied were similar to those reported for ANDV infections: fever, myalgia, headache, and vomiting, soon followed by pulmonary edema. Thrombocytopenia and hemoconcentration were reported, renal involvement was minimal, neither oliguria nor renal failure was observed in any case-patient, but all case-patients showed petechiae. The 4 HPS case-patients had immunoglobulin M (IgM) and IgG antibodies to ANDV N recombinant protein by ELISA (*13*) and all

Figure 1. Misiones Province, Argentina, and eastern Paraguay, where cases of hantavirus pulmonary syndrome have occurred and rodents were trapped for testing.

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survived. These cases led us to investigate reservoirs for hantaviruses in Misiones by using Sherman live-capture traps (H.B. Sherman Traps, Tallahassee, FL, USA). A total of 59 rodents were trapped at 2 study sites in Misiones, where *O. nigripes* was the most frequently captured rodent (42 specimens), followed by *Akodon montensis* (11 specimens). The rodent species was identified by morphologic features, in particular, qualitative external and cranial characteristics. Animals were tested for ANDV IgG antibodies (Table 1). The capture done in August 2005 in Santa Ana and Leandro N. Alem cities found 5 (11.9%) ANDV-positive *O. nigripes* rodents of 42 tested from this species.

Before we discovered the case in Itapua, we had conducted a serosurvey of rodents in 3 departments in eastern Paraguay (Table 1). Fifty-one rodent specimens were collected, of which 32 were sigmodontine rodents and 20 were *Akodon cursor*. Only 1 *A. cursor* rodent obtained from the Limoy Biologic Reserve, Paraguay, was seropositive. Because of the diversity of akodontine rodents, precise diagnosis, based solely on morphometric characteristics, is not always possible. To confirm the morphologic identification of the *A. cursor* rodent, we compared the mitochondrial control region (fragment of 245 nt) with that of an *A. montensis* rodent used as a control. Mitochondrial DNA sequencing of *A. cursor* from Limoy found higher identity (94%) with an *A. cursor* from Paraguay (AF296264) than with *A. montensis* (90%). Positive voucher specimens were archived at the Museo Argentino de Ciencias Naturales and at the University of Buenos Aires.

Amplification by reverse transcription–PCR was performed on the 4 human blood samples and on the lung tissues of the 6 seropositive rodents. Initially, a substantial portion of the nucleoprotein N coding region of the S segment (nt 50–954) and different fragments of the encoding region of the M segment: G1 glycoprotein (nt 41–443), G1–G2 glycoprotein (nt 1,728–1,976), and G2 glycoprotein (nt 2,715–2,941) were amplified and subsequently sequenced.

Comparison of the viral 905-nt N fragment sequence from case-patient 1 showed the highest degree of identity, ≈90%, with LEC (Table 2). The strains from case-patients 2 and 3 showed little genetic variation between them and were ≈95% identical to ARAV from Parana city, Brazil (*12*), in the same fragment. Comparison of a G1–G2 fragment available for JUQV strain (nt 1,867–1,976) with that from case-patient 2 showed a 93.6% identity. The G2 fragment from case-patient 3 was 95.6% identical to that of JUQV. Thus, these results suggest that the strains from case-patients 2 and 3 are JUQV, although they demonstrate that the strain called Araucaria would also be JUQV.

Sequences from the 5 positive *O. nigripes* from Misiones showed little variability between them and the N fragments were 98.3% identical to those in strains from case-patients 2 and 3. The case-patient from Itapua, Paraguay, showed the greatest nucleotide identity (93.5%) with BMJ

Table 1. Sigmodontine rodents captured and surveyed for antibodies to Andes hantavirus and viral RNA, northeast Argentina and eastern Paraguay*

Location	Period	Species	No.	Seropositive rodents, no (%)	RT-PCR–positive rodents
Caaguazu, Paraguay	Spring 2000	*Akodon* sp.	1	0	
		Calomys callosus	1	0	
Caaguazu, Paraguay	Summer 2001	*Holochilus brasiliensis*	4	0	
		C. callosus	1	0	
Central Paraguay	Summer 2001	*Oryzomys buccinatus*	2	0	
Reserve Limoy, Paraguay	Winter 2001	*A. cursor*	7	1 (14.3)	1
		C. callosus	2	0	0
Caaguazu, Paraguay	Spring 2001	*A. cursor*	8	0	
		A. nigrita	1	0	
Reserve Limoy, Paraguay	Summer 2002	*A. cursor*	5	0	
Leandro N. Alem, Misiones, Argentina	Autumn 2004	*Oligoryzomys* sp.	9	0	
		A. cursor	3	0	
		O. nigripes	1	0	
		O. flavescens	1	0	
		H. brasiliensis	1	0	
Santa Ana Misiones, Argentina	Winter 2005	*O. nigripes*	28	3 (10.7)	3
		A. montensis	8		
		O. flavescens	1		
Leandro N. Alem, Misiones, Argentina	Winter 2005	*O. nigripes*	14	2 (14.3)	2
		A. montensis	3	0	
		O. flavescens	3	0	
		C. laucha	2	0	

*RT, reverse transcription.

Table 2. Comparison of the nucleotide (first) and amino acid (second) sequences of the 905-nucleotide region of the N gene ORF among human and rodent hantavirus strains from South America with viruses from Misiones, Argentina, and eastern Paraguay*

	Case 1	Cases 2 ,3	Case Itapua	*Akodon cursor* Limoy	*O. nigripes*	LEC	BMJ	AND	JUQ	LN	ANAJ	SN
Case 1		82.2	91.2	74.1	82.1	96.5	92.3	83.8	82.5	77.6	80.1	76.4
Cases 2, 3	94.7		84.2	75.8	98.5	81.9	83.1	82.3	95.1	80.1	80.0	76.7
Case Itapua	99.3	94.0		74.0	84.0	90.8	93.5	83.5	84.9	78.7	81.0	75.9
Akodon cursor Limoy	86.1	85.4	85.7		75.7	74.8	74.7	75.7	75.9	74.7	76.9	73.9
Oligoryzomys nigripes	94.3	99.7	93.7	85.0		82.0	82.4	82.1	95.1	79.8	80.0	76.2
LEC	99.7	94.3	99.0	85.7	94.0		91.7	84.8	83.0	78.8	80.2	76.1
BMJ	100.0	94.7	99.3	86.0	94.3	99.7		84.3	83.5	79.0	79.9	76.8
AND	95.7	94.7	95.0	85.7	94.3	95.4	95.7		83.4	78.8	79.2	75.7
JUQ	94.7	100.0	94.0	85.4	99.7	94.4	94.7	94.7		80.6	80.7	76.4
LN	86.7	87.4	86.0	85.0	87.0	86.4	86.7	87.7	87.4		80.2	75.4
ANAJ	87.7	89.0	87.4	88.0	88.7	87.4	87.7	89.0	89.0	91.4		75.5
SN	85.4	84.1	85.0	84.0	84.0	85.1	85.4	83.7	84.1	82.4	82.4	

*ORF, open reading frame; LEC, Lechiguanas; BMJ, Bermejo; AND, Andes; JUQ, Juquitiba; LN, Laguna Negra; ANAJ, Anajatuba; SN, Sin Nombre. An expanded online version of Table 2 is available from www.cdc.gov/EID/content/13/8/1211-T2.htm

lineage, in the N fragment; identity at the amino acid level was 99.3%.

Another different strain was obtained from the *A. cursor* rodent captured in Limoy Reserve; this was the most distinct strain. The highest degree of identity exhibited was ≈77% (Table 2). The G1 fragment from this strain was compared with the closest related hantavirus and showed 67% identity with strains isolated from *O. nigripes*, case-patients 2 and 3, and LNV.

The N-encoding sequences were further subjected to phylogenetic analysis. All virus sequences from Misiones and the Itapua case form a monophyletic group together with ANDV lineages, nonpathogenic Pergamino (PRG) and Maciel (Figure 2), and CASV and ARAV (done in a 643-nt parsimonious tree, data not shown). The other South American clade was formed with Rio Mearim virus and Anajatuba virus from Brazil, together with LNV, HTN virus 007, and Rio Mamoré virus from Paraguay, Peru, and Bolivia, respectively, with a moderate support of 63%. Sequences from case-patients 2 and 3 from Misiones grouped together with JUQV (Araucaria) to form a separate clade, since they were from the most divergent strains. The sequence from case-patient 1 grouped with LEC lineage from Central Argentina. Different alignment parameters and phylogenetic methods produced the same results in trees with similar topology; however, bootstrap supports were moderate or low level for some lineages. The sequence from the *A. cursor* rodent was a quite

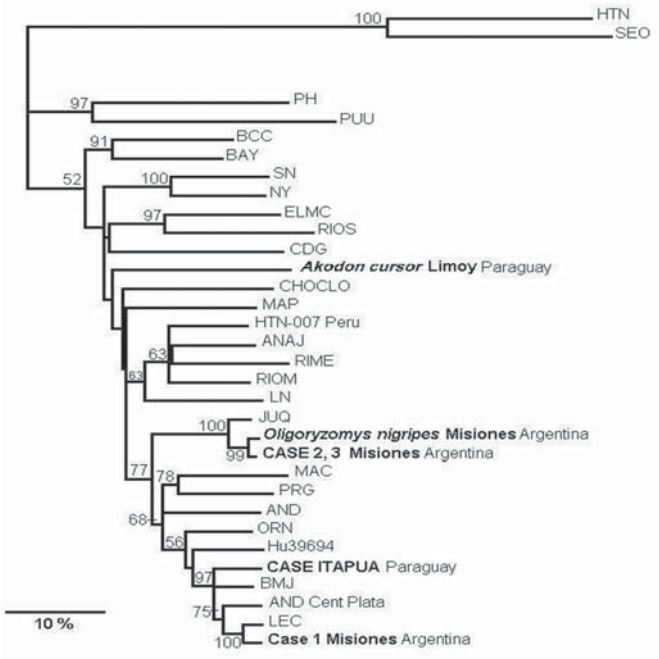

Figure 2. Phylogenetic relationships among the nucleotide sequences of the N protein of different hantaviruses from North America. A maximum parsimonious phylogenetic tree was generated on the basis of nucleotide sequence differences in the 904-nt region of the N gene open reading frame, which is available for South American strains by using PHYLIP version 3.57c. Bootstrap values >50%, obtained from 1,000 replicates of the analysis, are shown for the branch points. Lengths of the horizontal branches are proportional to the nucleotide step differences. The strain sequences under study in this paper are in italics. The following published S-segment sequences were included in the analysis (GenBank accession no.): Hantaan (HTN; U37768), Seoul (SEO; AB027522), Prospect Hill (PH; Z49098), Puumala (PUU; X61035), Black Creek Canal (BCC; L39949), Bayou (BAY; L36929), Sin Nombre (SN; L25784), New York (NY; U36801), El Moro Canyon (ELMC; U11427), Río Segundo (RIOS; U18100), Caño Delgadito (CDG; AF000140), Choclo (CHOCLO; DO285046), Maporal (MAP; AY267347), HTN-007 Perú (HTN-007 Perú. AF133254), Anajatuba (ANAJ; DQ451829), Rio Mearim (RIME; DQ451828), Río Mamoré Bolivia (RIOM; U52136), Laguna Negra (LN; AF005727), Araucaria (JUQ; AY740633), Maciel (MAC; AF0482716), Pergamino (PRN; 482717), Andes (AND; AF324902), Oran (ORN; AF028024), Hu39694 (Hu39694; AF482711), Bermejo (BMJ; AF482713), Lechiguanas (LEC; AF482714).

genetically distinguishable virus lineage, separated and apart from Choclo and Maporal viruses.

Conclusions

HPS is an emerging disease in South America, and investigations strengthen the belief that the disease is underestimated. Despite being surrounded by HPS-endemic countries, Misiones Province had no reported HPS cases until 2003. Furthermore, HPS cases have not been documented in eastern Paraguay. Two pathogenic hantaviruses that cause HPS have so far been proven to circulate in Misiones: LEC and JUQV. We confirmed *O. nigripes* as the reservoir species associated with 2 JUQV cases in Misiones. In eastern Paraguay, Itapua Department, BMJ lineage produced HPS. We have already reported a BMJ case in Bolivia (*7*). LEC was characterized originally from an *O. flavescens* mouse trapped in the Rio de la Plata River, an area where several HPS cases had occurred (*6*).

Species of *Akodon* are found throughout South America. To our knowledge, no *Akodon*-borne hantavirus has been reported to be associated with cases in South America. *A. azarae* is the most abundant sigmodontine species widely distributed in rural and peridomestic habits of central Argentina. In Buenos Aires Province, PRG was characterized in *A. azarae* populations. Despite the absence of reported HPS cases associated with this species in the studied area, a high seroprevalence, ≈10%, has been detected (*14*). We characterized a distinct *Akodon*-borne hantavirus at the Biologic Reserve of Paraguay, although we did not investigate whether this virus can produce illness. A prior study analyzed a collection of sigmodontine rodents from the major biomes of Paraguay where 1 *A. montensis* and 2 *O. nigripes* were positive for viral RNA (*15*). Precise identification of source populations in the reservoir and collection of quantitative data on their relative contribution to hantavirus transmission will be essential for disease control in the 3-country frontier.

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Migrating Birds and Tickborne Encephalitis Virus

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During spring and autumn 2001, we screened 13,260 migrating birds at Ottenby Bird Observatory, Sweden, and found 3.4% were infested with ticks. Four birds, each a different passerine species, carried tickborne encephalitis virus (TBEV)–infected ticks (*Ixodes ricinus*). Migrating birds may play a role in the geographic dispersal of TBEV-infected ticks.

Tickborne encephalitis is a viral zoonotic disease caused by the tickborne encephalitis flavivirus (TBEV). There are 3 subtypes of TBEV: the European subtype (TBEV-Eu, transmitted by *Ixodes ricinus* ticks) and the Siberian and Far-Eastern subtypes (TBEV-Sib and TBEV-FE, transmitted by *I. persulcatus* ticks) (*1*–*3*). Geographic distribution of TBEV subtypes largely follows that of their tick hosts: *I. ricinus* (Europe) and *I. persulcatus* (from Far East to the Baltic countries) (*4*). In Latvia and Estonia, the distribution of both tick species overlaps, and all 3 TBEV subtypes cocirculate in Latvia (*3*). Thus, a range expansion of a tick species could result in spreading a TBEV subtype to new areas.

Small rodents are thought to be the main amplifying hosts, although wild ungulates contribute indirectly by providing blood meals for adult ticks, thereby maintaining the vector populations necessary for virus transmission. In addition to mammals, *I. ricinus* ticks take blood meals from birds, which has led to speculation that birds could disperse TBEV-infected ticks during migration and start new TBE foci. In this study, we document the occurrence of TBEV-infected ticks in migrating birds.

The Study

Fieldwork was conducted during 2001 at Ottenby Bird Observatory, located on the southernmost tip of Öland, a large island off the southeast coast of Sweden (56° 12′ N, 16° 24′ E; Figure). Throughout spring (March 25–June 15) and autumn (July 1–November 15) migration, observatory personnel captured and screened birds for ticks, except during 8 days when an excessive number of trapped birds made complete monitoring impossible. Each captured bird was identified by species and age and was banded. For bird species with TBEV-infected ticks, local banding and recovery records from 1946 to the present were used to determine recruitment and wintering areas.

Tick screening comprised rapid visual assessment for the presence of any ticks on bare body parts, especially around the eyes and beak of each bird. All ticks were removed by forceps, placed separately into snap-lid tubes, frozen and stored at –70ºC, and then analyzed with a dissecting microscope to identify species and development stage.

A Puregene RNA isolation protocol adopted for 100–10,000 cells (Gentra Systems, Minneapolis, MN, USA) individually homogenized each tick and extracted RNA, according to the manufacturer's instructions. The RNA pellet was resolved in 25 µL DNA hydration buffer and stored at –70°C until further analysis.

Samples were pooled 10 by 10 (5 µL from each individual extract) and analyzed by a nested reverse transcription (RT)-PCR targeting the 5′-terminal noncoding region (*5*) for the initial detection of TBEV RNA. Briefly, the RT-PCR was performed in 25-µL reaction volumes containing 1× EZ buffer, 0.3 mmol of each deoxyribonucleotide

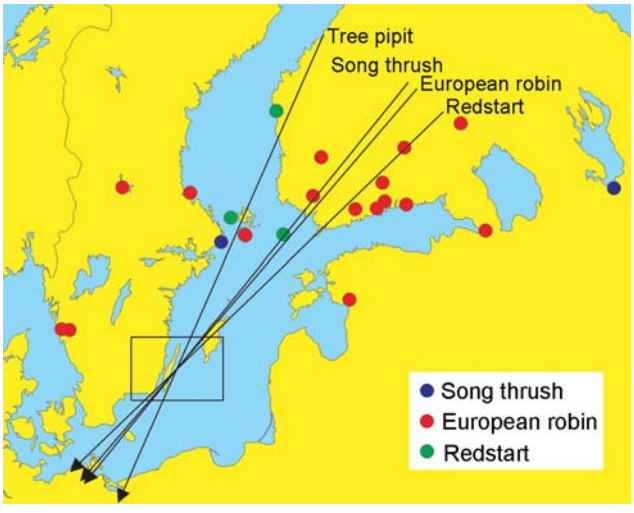

Figure. Autumn migration directions (arrows) of tree pipits (*Anthus trivialis*), robins (*Erithacus rubecula*), redstarts (*Phoenicurus phoenicurus*), and song thrushes (*Turdus philomelos*) banded in southeastern Sweden (area indicated by a square) and recovered within 60 days. Directions: Tree pipit, 203.6º, mean vector length = 0.993, n = 10; robin, 220.5º, mean vector length = 0.928, n = 293; redstart, 225.9º, mean vector length = 0.975, n = 52; and song thrush, 218.8º, mean vector length = 0.947, n = 117. Recovery sites of birds banded in southeastern Sweden and reported from areas north of the banding sites in a following year are also shown as indicated in the legend (no recovery from breeding areas is available for tree pipit).

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(dNTP), 2.5 U rTth DNA polymerase, 2.5 mmol $Mn(OAc)_2$ (all reagents provided from Perkin Elmer, Branchburg, NJ, USA), 25 pmol of each primer (Pp1 and Pm1), 25 U Rnasine (Gibco, Paisley, Scotland, UK), and the target viral RNA. The reaction was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) programmed to incubate 45 min at 60°C for RT and 2 min at 94°C for denaturation as initial steps, followed by

Table. Bird species infested with ticks during the spring and autumn migration periods

		Spring			Autumn		
Scientific name	Common name	No. infested	No. ticks	Infestation rate (ticks/infested bird)	No. infested	No. ticks	Infestation rate (ticks/infested bird)
Accipiter nisus	Eurasian sparrowhawk	–	–	–	1	2	2.0
Acrocephalus palustris	Marsh warbler	1	1	1.0	–	–	–
Acrocephalus scirpaceus	European reed warbler	–	–	–	1	1	1.0
Alauda arvensis	Eurasian skylark	–	–	–	1	6	6.0
Anthus trivialis	Tree pipit	1	1	1.0	10	28	2.8
Carduelis cannabina	Common linnet	1	1	1.0	–	–	–
Carduelis chloris	European greenfinch	6	6	1.0	1	1	1.0
Carduelis flammea	Common redpoll	–	–	–	1	1	1.0
Carduelis spinus	Eurasian siskin	1	1	1.0	–	–	–
Carpodacus erythrinus	Common rosefinch	–	–	–	1	1	1.0
Certhia familiaris	Eurasian treecreeper	–	–	–	2	2	1.0
Dendrocopos major	Great spotted woodpecker	–	–	–	1	8	8.0
Emberiza schoeniclus	Common reed bunting	–	–	–	1	1	1.0
Erithacus rubecula	European robin	35	58	1.7	153	404	2.6
Fringilla coelebs	Common chaffinch	1	1	1.0	1	8	8.0
Hippolais icterina	Icterine warbler	–	–	–	2	15	7.5
Lanius collurio	Red-backed shrike	–	–	–	2	7	3.5
Luscinia luscinia	Thrush nightingale	2	5	2.5	2	4	2.0
Luscinia svecica	Bluethroat	3	5	1.7	2	3	1.5
Parus caeruleus	Eurasian blue tit	2	3	1.5	4	11	2.8
Parus major	Great tit	2	2	1.0	18	34	1.9
Phoenicurus phoenicurus	Common redstart	3	6	2.0	9	18	2.0
Phylloscopus sibilatrix	Wood warbler	–	–	–	1	1	1.0
Phylloscopus trochilus	Willow warbler	3	3	1.0	16	18	1.1
Prunella modularis	Dunnock	2	6	3.0	2	3	1.5
Pyrrhula pyrrhula	Eurasian bullfinch	–	–	–	5	8	1.6
Regulus regulus	Goldcrest	–	–	–	1	1	1.0
Sturnus vulgaris	Common starling	–	–	–	10	19	1.9
Sylvia atricapilla	Blackcap	1	2	2.0	6	6	1.0
Sylvia borin	Garden warbler	–	–	–	1	1	1.0
Sylvia communis	Common whitethroat	6	13	2.2	12	33	2.8
Sylvia curruca	Lesser whitethroat	2	4	2.0	6	7	1.2
Sylvia nisoria	Barred warbler	2	3	1.5	1	1	1.0
Troglodytes troglodytes	Winter wren	7	16	2.3	11	19	1.7
Turdus iliacus	Redwing	8	17	2.1	2	5	2.5
Turdus merula	Common blackbird	24	86	3.6	20	89	4.4
Turdus philomelos	Song thrush	7	12	1.7	18	131	7.3
Turdus pilaris	Fieldfare	1	4	4.0	2	2	1.0
	Total	121	256		326	899	

40 cycles of 30 s at 94°C and 30 s at 66°C. The final extension was for 5 min at 66°C. Negative and positive controls were included in each PCR run.

A second amplification step was conducted with 2 μL of the first amplification products. The total reaction volume of 25 μL included 1× PCR buffer II, 1.5 mmol $MgCl_2$, 0.2 mmol each of dNTP, 0.625 U AmpliTaq Gold polymerase (Perkin Elmer), and 25 pmol of each internal primer (Pp2 and Pm2). After a pre-incubation step of 9 min at 95°C, the reaction was continued by 30 cycles of 15 s at 94°C and 30 s at 65°C and ended with an elongation step of 10 min at 72°C. Samples from positive pools were rerun using individual samples with the nested PCR described above.

During the study period, 1,155 ticks were collected from 447 (3.4%) of 13,260 screened birds (Table). Nearly all ticks (1,130) were reliably identified as *I. ricinus.* Seven nymphs showed characters resembling *I. lividus,* but these and 19 other ticks were rather poorly preserved, making identification uncertain. Frequencies of the various tick life stages were as follows: larvae (53.4%), nymphs (45.1%), and adults (0.6%). The mean infestation rate (0.086 immature ticks per examined bird, 2.6 immature ticks per infested bird) was unevenly distributed among bird species, with tick infestation in only 37 of >100 investigated species.

Ground-foraging birds carried ≈80% of all detected ticks and made up 71.3% of all infested birds (Table). A few ticks were also found on granivorous bird species, e.g., siskins, finches, sparrows, and some insectivorous songbirds, particularly among *Sylvia* and *Acrocephalus* warblers that forage in reed beds or dense stands of herbaceous plants (Table). The number of detected ticks per infested bird was usually in the range of 1–5 ticks, but 2 birds, a song thrush (*Turdus philomelos*) and a European robin (*Erithacus rubecula),* carried 41 and 39 ticks, respectively.

After initial screening of pools and rerunning individual samples from PCR-positive pools, we detected 6 TBEV-positive samples: 4 tick nymphs and 2 larvae. One larva was collected from a juvenile tree pipit (*Anthus trivialis)*, 1 nymph each from a song thrush and juvenile redstart (*Phoenicurus phoenicurus*), and 2 nymphs and 1 larva from a juvenile European robin. All TBEV-infected ticks were collected from birds during the autumn migration. Despite repeated trials, we were unable to obtain readable sequence data from the positive samples and could not identify the TBEV strains by subtype.

Conclusions

Our study found that some ticks attached to birds carried TBEV. However, the frequency of TBEV among such ticks was less than the frequency of *Borrelia burgdorferi* senso lato from similar datasets *(6–8*). Analyses of banding recovery data for the 4 bird species with TBEV-infected ticks indicate an eastern recruitment area coinciding with TBE-endemic areas in Fennoscandia and western Russia (Figure).

TBEV has been isolated, or serologically indicated, from several bird species, especially anatids and gallinaceous birds, and most often from Eastern Europe or Russia *(9)*. However, little is known about the capability of birds to function as reservoirs of TBEV, and small rodents remain the most important reservoirs of the virus. The fact that we found 2 *I. ricinus* larvae infected with TBEV could indicate that these birds may be reservoirs, because these larvae did not feed before attaching themselves to the birds. However, nonviremic transmission between ticks cofeeding on the same host has been shown to occur with TBEV *(10*) and other arboviruses *(11,12)*, and we did not look for viremia in the tick-infested birds.

The migration of birds through Scandinavia during spring and fall involves several hundred million birds. Although the tick infestation rate per bird was not great in our study, and TBEV-infected ticks were only a small fraction of all ticks, the vast numbers of migrating birds do increase the probabilities for geographic spread of ticks and TBEV, in particular for TBEV-Eu, because *I. ricinus* predominated in our sample. Our data add to the growing body of evidence showing that migratory birds can disperse ticks infected with medically important pathogens *(6,7,10,13)*.

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Avian Influenza (H5N1) Susceptibility and Receptors in Dogs

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Inoculation of influenza (H5N1) into beagles resulted in virus excretion and rapid seroconversion with no disease. Binding studies that used labeled influenza (H5N1) showed virus attachment to higher and lower respiratory tract tissues. Thus, dogs that are subclinically infected with influenza (H5N1) may contribute to virus spread.

Avian influenza (H5N1) virus has been shown to be infectious not only for birds but also for humans and mammals such as mice, ferrets, and cats. Carnivorous mammals that are susceptible to subtype H5N1 may contribute to spread of the virus; shedding of influenza (H5N1) by pet carnivores may pose a risk to humans. Cats experimentally inoculated with influenza (H5N1) have been shown to be susceptible to infection and to shed virus (*1*). However, dogs' susceptibility to this virus is unknown. Unpublished studies indicate that a substantial number of dogs tested in Thailand were positive for antibodies against H5N1 subtype (*2*). Recently, isolation of influenza (H5N1) virus from a dog in Thailand has been reported (*3*). We describe the susceptibility of specific pathogen–free (SPF) beagles to avian influenza (H5N1) and the presence of receptors for influenza (H5N1) in the respiratory tract of these dogs.

The Study

To study the infectivity of avian influenza (H5N1) in dogs, we inoculated 3 SPF beagles (HsdCpb:DOBE; Harlan Nederland, Horst, the Netherlands), 16 weeks of age, with 10^6 median tissue culture infectious doses of influenza (H5N1) (A/chicken/GxLA/1204/2004). Half of the dose (0.5 mL) was administered intranasally and the other 0.5mL intratracheally. Body temperature and health status of the dogs were monitored twice a day during the first 5 days after challenge and once a day thereafter. No major changes in body temperature and no clinical signs were noted.

Excretion of virus was monitored daily in swabs from rectum, oropharynx, and nose. The presence of influenza (H5N1) virus in these swabs was studied by inoculation into embryonated eggs and by real-time reverse transcription–PCR that targeted the matrix gene. For the PCR, we used the forward primer AI-M-F45 (5′-CTTCTAACCGAGGTCGAAACGTA-3′, reverse primer AI-M-R251 (5′-CACTGGGCACGGTGAGC-3′) and Taqman probe AI-M-Tqmn1 (5′-6FAM-CTCAAAGCCGAGATCGCGCAGA-XT-PH) (TIBMolBiol, Berlin, Germany). A calibration curve consisting of serial dilutions of a standard batch of influenza (H5N1) virus with a known median 50% egg infectious dose (EID_{50}) titer was included in each PCR. One of the dogs shed virus for several days after challenge. In this dog, virus was demonstrated by PCR in nasal swabs taken on days 1 through 4 after challenge and by virus isolation in embryonated chicken eggs on days 2 and 3 after challenge (Table 1). Quantification by real-time PCR indicated that the amount of virus present in the nasal swabs corresponded to 2.0–3.2 $\log_{10}$ EID_{50}.

Serum samples collected at days 7 and 14 after challenge were tested for antibodies against H5N1 subtype in an influenza A nucleoprotein-blocking ELISA as well as in a hemagglutination-inhibition assay. An antibody response against influenza (H5N1) was detectable in 1 dog at day 7 after challenge. In all dogs an antibody response against influenza (H5N1) was demonstrated in both assays at day 14 after challenge (Table 2). Postmortem examination on day 14 after virus challenge showed no gross pathologic or histopathologic changes in the respiratory tract and other organs. Considering the time of sampling after challenge, transient histopathologic changes may have occurred unnoticed.

Influenza (H5N1) viruses bind with their hemagglutinin surface proteins to cell surface oligosaccharides terminating in sialic acid α2,3 galactose (SA2,3Gal) (*4*). In humans and cats, influenza (H5N1) virus predominantly attaches to

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Table 1. Virus detection in nasal swabs from dogs inoculated with avian influenza (H5N1)*

	Days postinoculation							
	1		2		3		4	
Dog	PCR†	Egg	PCR	Egg	PCR	Egg	PCR	Egg
1	$10^{3.2}$	–	$10^{2.0}$	+	$10^{2.9}$	+	$10^{2.8}$	–
2	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–

*PCR, real-time PCR of nasal swabs from dogs; Egg, embryonated chicken eggs.; + virus detected; –, virus not detected.
†After quantification by PCR, virus titers are expressed as 50% egg infectious dose.

Table 2. Antibody titers in serum of dogs inoculated with avian influenza (H5N1)*

	Days postinoculation					
	0		7		14	
Dog	ELISA	HI†	ELISA	HI†	ELISA	HI†
1	–	–	–	–	+	16
2	–	–	–	–	+	16
3	–	–	+	16	+	32

*Antibodies were measured in a nucleoprotein-blocking ELISA and in the hemagluttination-inhibition (HI) assay at different days postinoculation. +, antibodies detected; –, antibodies not detected.
†HI titers are presented as the reciprocal to the highest serum dilutions completely inhibiting agglutination of chicken erythrocytes by influenza (H5N1) virus.

the lower part of the respiratory tract where the SA2,3Gal receptors are present (*5*). To study the attachment pattern of influenza (H5N1) in the respiratory tract of dogs, we performed binding experiments with labeled influenza (H5N1) virus on formaldehyde-fixed and paraffin-embedded tissue sections. We collected tissues directly from a euthanized control dog of the same breed as that used for the infection experiment and fixed them in 4% buffered formaldehyde solution for several weeks. Virus labeling and histochemical examination were performed according to van Riel et al. (*5*). Briefly, influenza (H5N1) virus was grown in 10-day-old embryonated eggs and inactivated with formalin. After being purified by sucrose gradient centrifugation, the virus was labeled with fluorescein isothiocyanate (FITC). Staining was performed by incubating tissue sections with FITC-labeled virus and detecting the FITC-label with a peroxidase-labeled rabbit anti-FITC. To create a negative control, staining was performed without prior incubation with labeled virus or after prior incubation with FITC alone. No staining was observed in these negative controls. Chicken tissues were used as positive controls.

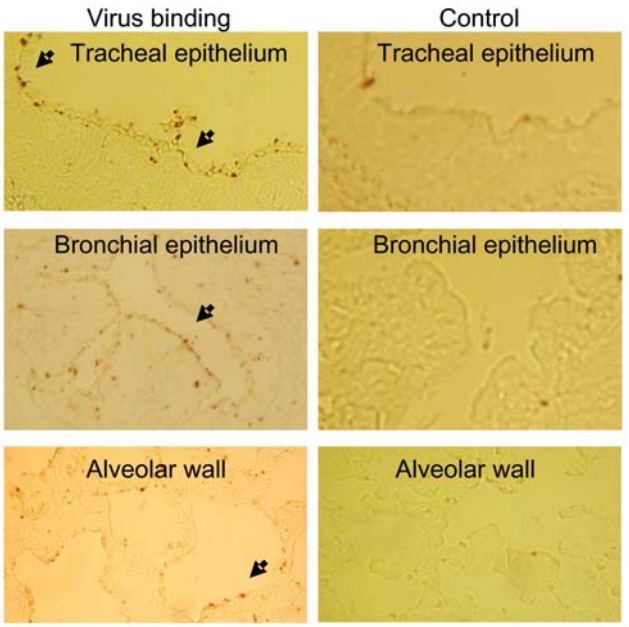

Figure. Binding of fluorescein isothiocyanate–labeled influenza (H5N1) virus to formaldehyde-fixed, paraffin-embedded tissue slides of dog respiratory tract tissues. Left panel shows binding of virus (arrow). Right panel shows blocking of virus binding by competitive binding of *Maackia amurensis* lectin to sialic acid α2,3 galactose.

Strong virus binding to the epithelia of the chicken respiratory tract tissues was noted. Furthermore, as a specificity control, preincubation with *Maackia amurensis* lectin, which specifically binds to SA2,3Gal–terminated oligosaccharides, was performed. Moderate particulate binding of influenza (H5N1) to canine nasal mucosa, tracheal epithelium, and alveoli was observed. Strong multifocal binding was observed in bronchial epithelium (Figure). This staining pattern was also found after binding of FITC-labeled *M. amurensis* lectin to canine respiratory tract tissues. Virus binding could be blocked with unlabeled *M. amurensis* lectin.

Conclusions

Our results demonstrate that dogs are susceptible to infection with avian influenza (H5N1) virus and can shed virus from the nose without showing apparent signs of disease. Moreover, receptors for avian (H5N1) virus are present not only in the lower part of the respiratory tract of dogs but also in their trachea and nose, which are potential portals of entry for the virus.

Influenza virus infection of dogs was first reported in 2004 (*6*). Influenza (H3N8) of equine origin caused outbreaks in greyhounds in Florida and has since been found in dogs in >20 US states (*7*). The course of experimental infection of SPF dogs with subtype H5N1 resembles that of the experimental infection of dogs with the subtype H3N8 (*6*): all dogs seroconverted, and some excreted virus without obvious disease. In contrast to the experimental outcomes, natural infections with influenza (H3N8) resulted in serious illness, death, and widespread infection for dogs. This finding warrants special attention to the potential course of avian influenza (H5N1) infection in dogs. Therefore, dogs' contact with birds and poultry should be avoided in areas with influenza (H5N1) outbreaks to prevent possible spread of virus and human exposure to influenza (H5N1) virus that might have been adapted to mammals.

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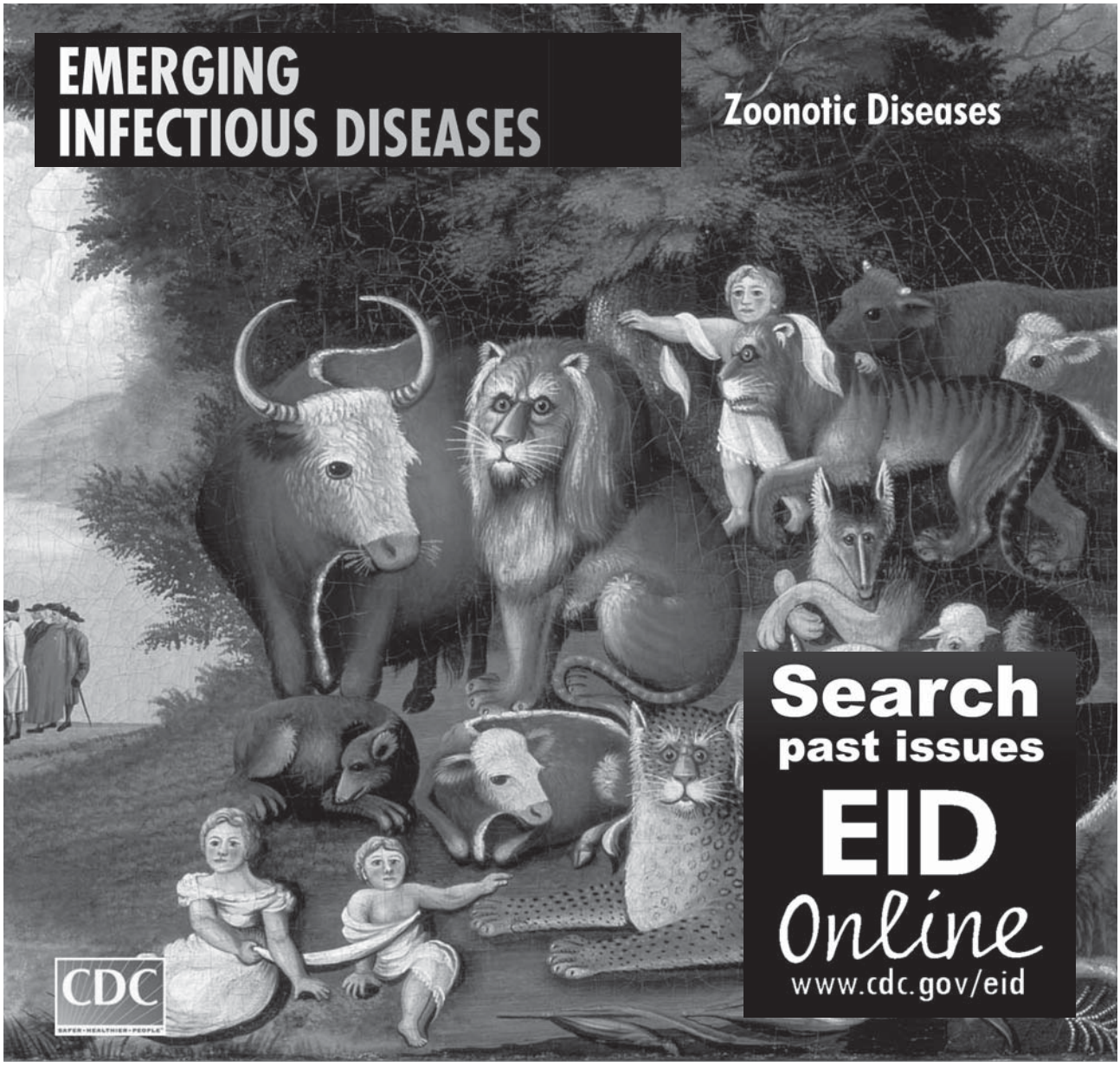

Molecular Epidemiology of Canine Parvovirus, Europe

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Canine parvovirus (CPV), which causes hemorrhagic enteritis in dogs, has 3 antigenic variants: types 2a, 2b, and 2c. Molecular method assessment of the distribution of the CPV variants in Europe showed that the new variant CPV-2c is widespread in Europe and that the viruses are distributed in different countries.

Canine parvovirus type 2 (CPV-2) is a small, nonenveloped DNA virus that emerged suddenly in 1978 as an enteric pathogen of dogs. Two antigenic variants, CPV-2a and CPV-2b, are now distributed worldwide (*1*). A third CPV variant, first named Glu-426 mutant and subsequently renamed CPV-2c, was detected in Italy in 2000 (*2*) and is now circulating in that country together with types 2a and 2b (*3*–*5*). The new variant 2c has also been reported in Vietnam by Nakamura et al., who developed monoclonal antibodies that can identify specifically such a mutant (*6*) and more recently in the United States by Saliki et al. (unpub. data) and in South America (*7*). The antigenic variants differ from the original type CPV-2 for a few amino acids in the VP2 protein, whereas genetic differences among the variants are determined only by residue 426, with types 2a, 2b, and 2c displaying Asn, Asp, and Glu, respectively (*8*,*9*). Recently, minor groove binder (MGB) probe assays have been established for characterization of CPV strains and account for the presence of single nucleotide polymorphisms in the genome of the different variants (*5*). Our objective was to determine the distribution of the CPV variants in different European countries by using the new technology, with particular emphasis on the widespread circulation of CPV-2c in some areas of Europe.

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The Study

A total of 232 fecal samples or CPV isolates were obtained from dogs with diarrhea in Italy (n = 107), Germany (n = 37), the United Kingdom (n = 41), Portugal (n = 31), Belgium (n = 13), Spain (n = 1), Switzerland (n = 1), and the Czech Republic (n = 1). The fecal samples collected in Italy, the United Kingdom, Portugal, and Belgium were CPV-positive according to PCRs performed in local laboratories. Samples were collected during 2005–2006, with the exception of samples from Italy that were collected only in 2006 because other studies had assessed the molecular epidemiology of CPV in the previous decade (*5*). All samples from Germany consisted of cell-culture–adapted CPV strains isolated from dogs with diarrhea in Germany during 1996–2005.

Samples were homogenized (10% w/v) in phosphate-buffered saline (pH 7.2) and subsequently clarified by centrifuging at 1,500× *g* for 15 min. Viral DNA was extracted from the supernatants of fecal homogenates or from the viral suspensions by boiling for 10 min and chilling on ice. To reduce residual inhibitors of DNA polymerase activity to ineffective concentrations, the DNA extracts were diluted 1:10 in distilled water (*10*). CPV DNA titers were calculated by using a real-time PCR, based on TaqMan technology and able to recognize all CPV strains (*10*), whereas characterization of the viral type was obtained by means of MGB probe assays specific for types 2a/2b and 2b/2c (*5*). To rule out the presence of CPV strains of vaccine origin, which are usually type 2 or less frequently type 2b, we subsequently tested samples recognized as types 2/2a and 2b by MGB probe assays that discriminate between vaccine and field strains of CPV (*11*,*12*).

All samples that had CPV-positive results from laboratories located in the countries of sample origin were confirmed by TaqMan assay to contain CPV DNA. CPV infection was also demonstrated in the single samples from Spain, Switzerland, and the Czech Republic (Figure). In Italy, a nearly complete substitution of CPV-2b by CPV-2c was noted; CPV-2a strains were found at low frequency, which confirmed the progressive decrease noted during the past 5 years (*3*–*5*). In contrast, CPV-2b is still prevalent in Germany and Portugal, although CPV-2c is also widespread in these countries. Retrospective analysis of archival samples showed that CPV-2c has been circulating in Germany since 1996. An equivalent distribution of types 2a and 2b was assessed in the United Kingdom, where a single CPV-2c strain was detected. In Portugal, no CPV-2a strain was detected, whereas in Belgium all CPVs were type 2a. The single samples from Switzerland and the Czech Republic were CPV-2a, whereas the sample from Spain was CPV-2c. The original type CPV-2 was detected in 1 sample from the United Kingdom and 4 from Italy, but the latter samples had been collected from dogs shortly after vaccination with a classic type-2–based vaccine (*12*).

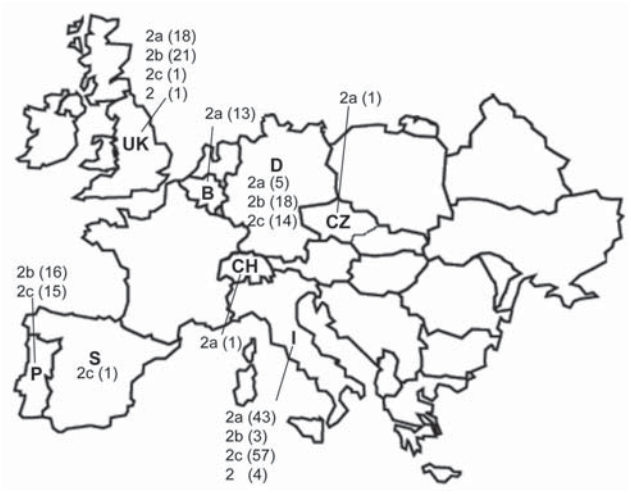

Figure. Geographic distribution of antigenic variants of canine parvovirus (CPV) in Europe. CPV-positive fecal samples or viral isolates from different countries were analyzed by molecular methods; strains CPV-2, 2a, 2b, or 2c are indicated for each country by numbers in parentheses. Samples were collected during 2005–2006, except for samples from Italy (2006) and Germany (1996–2005). P, Portugal; S, Spain; UK, United Kingdom; B, Belgium; CH, Switzerland; D, Germany; CZ, Czech Republic.

Conclusions

Despite its DNA genome, CPV possesses a high genetic substitution rate, similar to that observed for RNA viruses, which is responsible for continuous antigenic evolution and rapid displacement of old types by new antigenic variants (*13*). CPV-2c, which emerged in Italy in 2000 (*2*), is spreading with high efficiency in the dog population of Italy and progressively replacing the antigenic variants 2a and 2b (*3*–*5*). Such a variant has been detected in Vietnam also (*6*), but no information is available on its presence and distribution in other European countries, except for a single case report from Spain (*14*).

Our study shows that the new variant 2c is widespread in some European countries (Italy, Portugal, and Germany) and that it could be detected sporadically in the United Kingdom. In contrast with previous studies of CPV-2a in Europe, our study showed that CPV-2a is most frequent in Belgium, whereas in the United Kingdom, Germany, and Italy, it has been overtaken by CPV-2b or CPV-2c. In Portugal, CPV-2a was not detected at all, but types 2b and 2c were equally distributed. To our knowledge, this is the first report of the new variant 2c in Portugal, the United Kingdom, and Germany. In Germany, CPV-2c was detected in archival samples collected in 1996; thus, it was circulating in Europe 4 years before its first official report in Italy. Such a variable geographic distribution of the CPV variants in Europe may be related to different commercial flows of dogs imported from foreign countries rather than to different vaccination protocols.

The progressive spreading of CPV-2c in the world or, less probably, its independent emergence in different countries, suggests that the Glu-426 mutation provides a certain advantage in viral replication. The replacement of CPV-2 by types 2a and 2b has been associated with increased ability to bind canine transferrin receptors, although it might not rule out the possibility of mutations at residue 426 of the VP2 protein being selected also for their antigenic effects (*1*). Whether the Glu-426 mutation confers benefit in receptor-binding activity is of interest. Another question is whether the CPV vaccines currently used provide full protection against the new variant or whether they should be replaced by homologous vaccines.

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Dr Decaro is an associate professor at the Faculty of Veterinary Medicine of Bari, Italy. His research interests include the study of viral pathogens of dogs, with particular emphasis on canine parvovirus and canine coronavirus.

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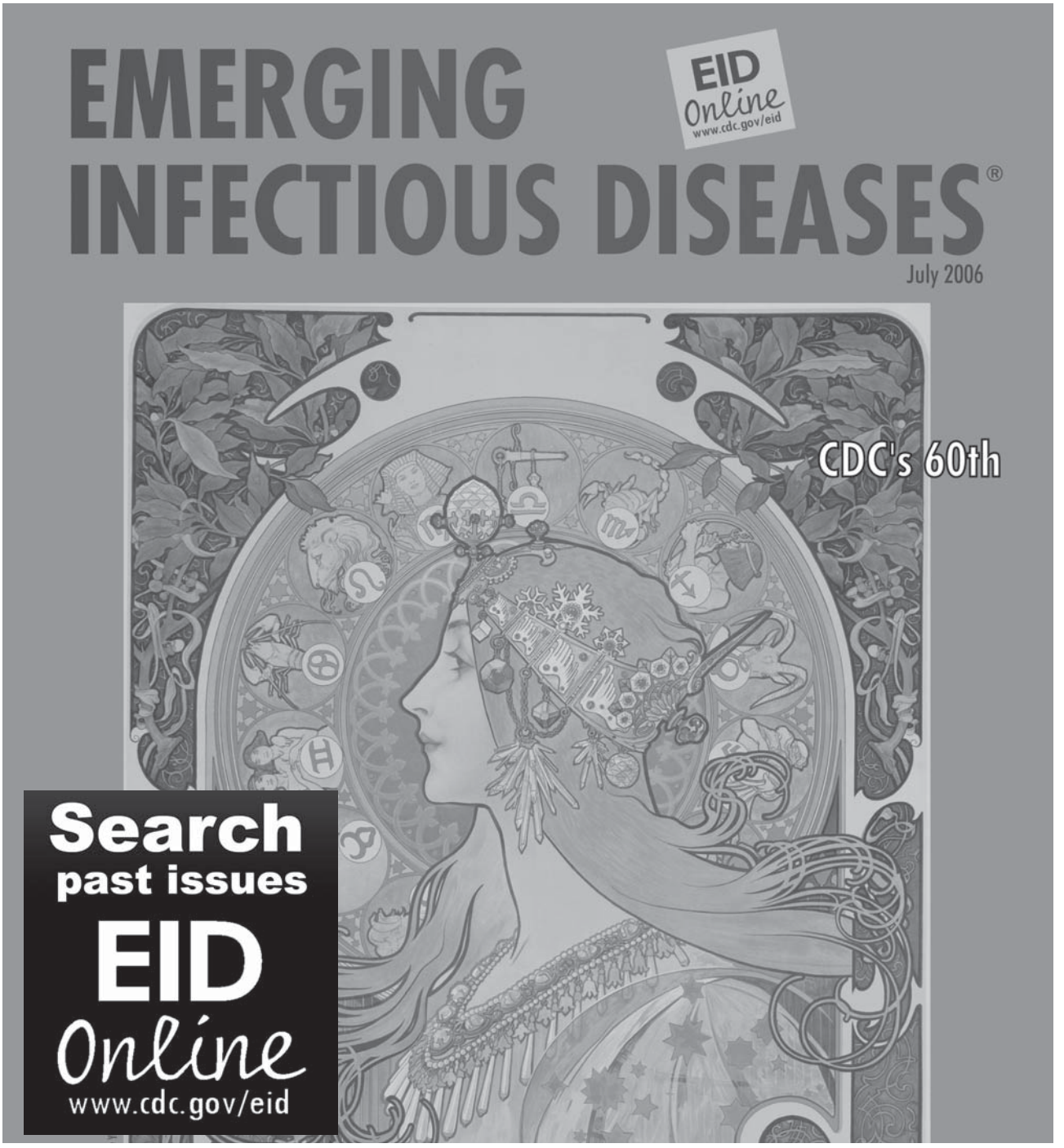

Invasive Meningococcal Disease, Utah, 1995–2005

Rachelle B. Boulton,*† Stephen C. Alder,* Susan Mottice,† A. Peter Catinella,* and Carrie L. Byington†

Trends in invasive meningococcal disease in Utah during 1995–2005 have differed substantially from US trends in incidence rate and serogroup and age distributions. Regional surveillance is essential to identify high-risk populations that might benefit from targeted immunization efforts.

Invasive meningococcal disease (IMD) refers to the many illnesses caused by infection with *Neisseria meningitidis*. IMD is an immediately reportable disease in Utah and a nationally reportable disease in the United States. A preliminary review of IMD in Utah suggests that, since 2000, epidemiologic trends have occurred that are distinct from trends reported elsewhere in the United States. We describe the change in incidence rates, serogroup distribution, and age distribution of IMD in Utah, based on cases reported from 1995 through 2005, and compare our results with US trend data from the same period.

The Study

We studied cases of IMD that occurred from January 1, 1995, through December 31, 2005, and were reported to the Utah Department of Health. Cases were classified as confirmed, probable, or suspected, based on the case definition for *N. meningitidis* infection in the Centers for Disease Control and Prevention and the Council of State and Territorial Epidemiologists 2005 case definition guidelines for IMD (*1*). Suspected cases, in which an isolate was not obtained, were not included in the final analysis because this study emphasized serotyping.

Utah incidence rates were calculated by using population estimates determined by Utah's Indicator-Based Information System for Public Health (*2*). Incidence rates and serogroup distributions published in the *N. meningitidis* Active Bacterial Core surveillance (ABCs) reports were used to estimate US trends (*3*).

The Pearson χ^2 test and Fisher exact test were used to test the statistical significance of the prevalence of serogroups by period for Utah and US data. Statistical analysis was performed with SAS (version 9.1; SAS Institute, Cary, NC, USA).

In the 10-year study period, 128 reported cases met the criteria of either confirmed or probable. Yearly incidence rates were calculated and ranged from a high of 0.95/100,000 population/year to a low of 0.21/100,000 population/year (Figure). Because the number of annual cases dropped after 1999, the data were divided into 2 periods. The mean incidence rate decreased significantly, by 63%, from 0.80/100,000 population/year from 1995 through 1999 (hereafter period 1) to 0.30/100,000 population/year from 2000 through 2005 (hereafter period 2).

Incidence rates by period were stratified by age (Table 1). A rate difference was calculated by subtracting the average incidence rate for period 1 from the average incidence rate for period 2. The highest rate for both periods was for infants <1 year of age (period 1, average incidence rate 7.98/100,000 population/year; period 2, average incidence rate 3.07/100,000 population/year). The greatest rate difference also occurred for this age group, a decrease of 4.91/100,000 population/year between the 2 periods' mean incidence rates (Table 1).

The serogroup distribution in Utah changed substantially over the course of the 2 study periods. Before 2000, Utah meningococcal serogroup distribution reflected that of the United States; that is, serogroups B, C, and Y each caused ≈30% of IMD (*4*). Beginning in 2000, however, the percentage of serogroup B infections in Utah decreased significantly to 11.3%, while serogroup Y infections increased to 50.0% ($p = 0.0102$, Fisher exact test; $\chi^2 = 7.2562$, $p = 0.0071$). A similar change was not seen in US data. Whereas no significant difference was observed between Utah and ABCs data during period 1, a significant difference was seen for serogroups B ($p = 0.0002$) and Y ($p < 0.0001$) when period 2 data were compared (Table 2). Because of an ongoing outbreak of serogroup B disease in Oregon, Utah data were compared with US data both with

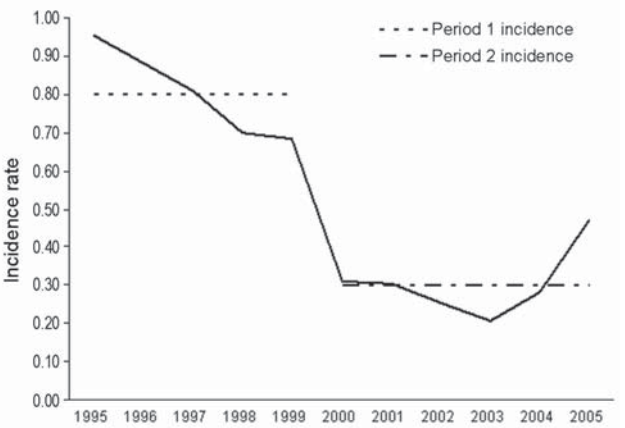

Figure. Incidence of invasive meningococcal disease by year, Utah, 1995–2005.

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Table 1. Rates of invasive meningococcal disease by age group, Utah, 1995–2005*

Age, y	No. period 1 cases	No. period 2 cases	Period 1 rate/100,000	Period 2 rate/100,000	Rate difference
<1	17	9	7.98	3.07	–4.91
1–4	14	4	1.75	0.36	–1.39
5–14	10	4	0.53	0.17	–0.37
15–24	20	15	1.03	0.58	–0.45
25–34	4	3	0.25	0.26	0.01
35–44	6	2	0.40	0.11	–0.29
45–54	4	2	0.39	0.13	–0.26
55–64	3	2	0.48	0.20	–0.27
65–74	3	1	0.61	0.16	–0.46
75–84	2	1	0.66	0.24	–0.42
≥85	1	1	1.03	0.68	–0.35
Total	84	44	0.80	0.30	–0.50

*Period 1, 1995–1999; period 2, 2000–2005.

and without Oregon's numbers. For both comparisons, the conclusions were the same, and therefore Oregon's numbers were not removed from the final analysis.

No Utah cases identified during the study period involved residents of military barracks or college dormitories, in which an increased risk for meningococcal disease is well documented (*5*,*6*). However, 5 (3.9%) patients were residents at a Job Corps facility (a residential job-training facility for young adults similar to a college dormitory). Of the 5 Job Corps cases, all were caused by serogroup Y infection, and 3 patients had bacteremic pneumonia.

The reduction in incidence rate could have several possible causes. One such cause could be a systematic change in reporting. However, no evidence to support this conclusion was found. Although the total number of reported cases declined between the 2 periods for most reporting hospitals, no single decline was strong enough to account for the observed decrease in reported cases. Underreporting of cases is another possible cause, but also is unlikely. Data from cases of IMD reported to the Utah Department of Health with onset dates from January 1, 2002, through December 31, 2005, were compared with data extracted from computerized laboratory records of a large hospital corporation in Utah for the same period. Ten cases of IMD were identified in each system, and demographic information confirmed that they were the same 10 patients.

Vaccination is unlikely to be the cause of the reduction in incidence rate as well. Over the study period, the percentage of vaccine-preventable strains causing disease in Utah increased, while infections caused by serogroup B, which is not included in the vaccine formula, decreased. Additionally, the greatest decrease in age-specific incidence rates occurred in age groups for which vaccination was not indicated.

Therefore, the decrease in the incidence rate seen is most likely the result of fluctuations in the community incidence rate, for which oscillations with a cyclical pattern have been documented (*7*–*9*). The incidence rate of IMD in Utah in 2005 increased substantially from the rate observed in 2004 (Figure). Although this rate is still much lower than rates seen for any year in period 1, it is still much greater than any other rate observed in period 2; the incidence rate appears to be increasing again, while the serogroup distribution is not changing. Due to the cyclical pattern of meningococcal disease, variability is expected, but the increase in serogroup Y cases and decrease in serogroup B cases appear unique to Utah.

Conclusions

During the second study period (2000–2005), the incidence rate and age and serogroup distributions for IMD in Utah have differed from US trends. In Utah, the decrease in serogroup B infections, the most common cause of IMD in infants, resulted in an overall decrease in infections in infants and increased infection rates in adolescents and young adults ages 15 to 24 years. Furthermore, of the 38 serogrouped isolates from period 2, 31 (82%) were vaccine-preventable strains. This suggests that recommendations by the Advisory Committee on Immunization Practices (ACIP) for routine vaccination of selected cohorts

Table 2. Serogroup distribution of invasive meningococcal disease by period*

Neisseria meningitidis serogroup	Period 1			Period 2		
	Utah	United States	p value	Utah	United States	p value
B	32.1%	29.2%	0.5701	11.3%	39.7%	0.0002
Y	26.1%	32.0%	0.2773	50.0%	23.9%	<0.0001
C	25.0%	25.5%	0.9298	15.9%	21.4%	0.3783
Other	16.7%	13.4%	0.4084	22.7%	15.0%	0.1633

*Period 1, 1995–1999; period 2, 2000–2005. US data estimates based on information collected from Active Bacterial Core surveillance sites.

with meningococcal conjugate vaccine (MCV-4) would be beneficial in Utah. ACIP recommendations, however, may not reflect regional epidemiologic trends. For example, Job Corp residents were identified as a high-risk population for IMD in Utah but have not been identified as a high-risk group in the United States. Because IMD is so rare, routine vaccination is costly (*10*), and vaccine supply is limited, we believe regional surveillance is a key factor in determining groups at high risk for IMD. The identification of serogroup Y disease among Job Corps residents influenced Utah's vaccine policy. This study emphasizes the need for continued regional surveillance to help direct vaccine policy especially in regions of the United States not represented in ABCs.

Acknowledgments

We are indebted to the nurses and epidemiologists at local health departments in Utah and health information services staff at many hospitals for their help in obtaining data for this study. We also thank the Utah Public Health Laboratory for their assistance in gathering serogoup data and the staff of the Communicable Disease Epidemiology Program at the Utah Department of Health.

Ms Boulton is an epidemiologist at the Utah Department of Health. Her interests include vaccine-preventable disease epidemiology and outbreak investigation.

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Outbreak of Sporotrichosis, Western Australia

Kynan T. Feeney,* Ian H. Arthur,† Amanda J. Whittle,‡ Shelley A. Altman,† and David J. Speers†

A cluster of sporotrichosis cases occurred in the Busselton-Margaret River region of Western Australia from 2000 to 2003. Epidemiologic investigation and mycologic culture for *Sporothrix schenckii* implicated hay initially distributed through a commercial hay supplier as the source of the outbreak. Declining infection rates have occurred after various community measures were instigated.

Sporotrichosis is an infection caused by the fungal species *Sporothrix schenckii*. It predominantly causes subacute to chronic subcutaneous infection, which occurs when the fungus enters small breaks in the skin (*1*). *S. schenckii* is widely found on organic material in the environment such as sphagnum moss, fruits, and plants. Human cases have been reported from intermediate hosts; cats were the source of an outbreak in Rio de Janeiro (*1*–*3*). Outbreaks in Australia and other countries have been previously linked to contact with hay (*4*,*5*).

In Western Australia, sporadic cases have occurred for many years in the southwest, particularly in the wheat-growing areas (Figure 1), but in the year 2000 an increase in the number of cases of sporotrichosis was noted (*6*). Forty-one microbiologically confirmed human cases were reported from 2000 to 2003 compared with 8 cases from 1997 to 1999 at the PathWest Laboratory at QEII Medical Centre, which has branches throughout metropolitan and regional Western Australia. A review of these cases found that 22 cases were from the Busselton-Margaret River (BMR) region of Western Australia, where no cases had previously been recorded.

The Study

An epidemiologic study was begun in 2003 to determine the nature and source of the infection. Pathology laboratories and general practitioners in the southwest region of Western Australia were contacted to ascertain patients with cases of sporotrichosis, defined as clinical evidence of disease supported by microbiologic confirmation. Telephone interviews, which included questions on possible sources of infection, were conducted with all identified patients.

The epidemiologic study (Table 1) discovered 11 patients with a microbiologic diagnosis of sporotrichosis from July 2003 to July 2004 in the southwest region. All lived in the BMR region except for 2 (patients 3 and 5).

Nine of the 11 case-patients had contact with hay preceding the development of sporotrichosis; 8 had bought the hay from 1 of 2 commercial suppliers located in the township of Margaret River (outlet 1 and outlet 2). The remaining patient had contact with hay grown on his property. Seven of the 9 persons with cases linked to hay exposure had used the hay for domestic gardening; the other 2 patients were exposed to hay used for commercial farming. Patients 10 and 11 were children who played together in hay purchased from outlet 1 before the onset of symptoms of sporotrichosis. Patients 1 and 3 had no documented hay exposure; sporotrichosis developed after a camping trip and after gardening, respectively.

Nine case-patients were initially treated with oral antimicrobial agents for a presumed bacterial infection. Because of a lack of clinical response, wound swabs or biopsy specimens were obtained. Sporotrichosis was subsequently diagnosed, and antifungal therapy was then begun. Four patients required inpatient treatment for 1 of several reasons: initially to receive intravenous antimicrobial agents before the diagnosis, to undergo debridement surgery and biopsy, or to treat complications related to

Figure 1. Wheat-growing region and Busselton-Margaret River region of Western Australia.

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Table 1. Sporotrichosis cases in Western Australia, July 2003–July 2004

Patient no.	Sex	Age, y	Area of residence	Pre-existing cuts	Onset of symptoms, mo/y	Duration of disease, mo	Antifungal treatment	Suspected exposure*	GenBank accession no.
1	F	57	BMR†	Present	7/03	6	Surgery, oral traconazole, oral K‡	Camping trip in a different region of Australia	EF589115
2	M	48	BMR	Present	10/03	5	Oral traconazole	Hay (2)	EF589117
3	M	68	Greenbushes	Present	11/03	4	Oral traconazole	Gardening	EF589116
4	F	39	BMR	Present	12/03	4	Oral traconazole	Hay (1)	EF589119
5	M	65	Collie	Present	12/03	4	Oral traconazole	Hay (own supply)	Not available
6	M	56	BMR	Absent	12/03	4	Oral traconazole	Hay (1)	EF589121
7	F	69	BMR	Absent	1/04	2	Oral traconazole	Hay (1)	EF589118
8	M	66	BMR	Absent	1/04	3.5	Oral traconazole	Hay (2)	EF589120
9	M	10	BMR	Present	2/04	2	Surgery, oral traconazole	Hay (1)	EF589122
10	F	8	BMR	Present	7/04	5	Oral traconazole	Hay (1)	EF589124
11	F	3	BMR	Present	7/04	5	Naturopathic treatment	Hay (1)	EF589123

*1 and 2 refer to outlet 1 and 2, respectively.
†Busselton-Margaret River region.
‡Saturated solution of potassium iodide.

therapy. Ten of the 11 patients received oral itraconazole; 1 of these patients also received a saturated solution of potassium iodide, and 1 patient ceased oral antifungal therapy because of severe side effects. One of the 11 received only a naturopathic remedy.

When hay was implicated as a likely source, local environmental health officers visited commercial hay outlets in the area to assess procurement, storage, and distribution practices. Fifty hay samples were collected from around the BMR region for mycologic culture. Sixteen samples were from properties associated with 6 of the cases, 9 from the 2 commercial hay outlets, 7 from a farm that supplied hay to a commercial outlet, and 18 from other properties in the BMR region not associated with the outbreak (control samples).

Mycologic culture for organisms from the *Ophiostoma–Sporothrix* complex was performed on a portion of each hay sample. Isolates were examined for morphologic features and analyzed by pulsed-field gel electrophoresis (PFGE) (*7*). Sequencing the ITS1 region with universal primers *ITS1* and *ITS2* and the ITS2 region with universal primers *ITS3* and *ITS4* was performed with Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing reagent using the Applied Biosystems Prism 3100 Avant Genetic Analyzer (Foster City, CA, USA). Using ITS sequencing, de Beer et al. (*8*) distinguished clinical strains of *S. schenckii* from environmental strains.

Isolates positive for the clinical group of *S. schenckii* showed morphologic features of conidia that are predominantly oval and display sleeves of dematiaceous conidia along the hyphae. When BLAST software (www.ncbi.nlm.nih.gov/blast) was used, these isolates had ITS2 sequences that conform to the clinical group noted by de Beer et al. (*8*). They differ from Western Australian environmental isolates of the *Opiostoma–Sporothrix* complex obtained in this study and clinical *S. schenckii* isolates from the eastern states of Australia in their morphologic features, PFGE patterns (*7*), and ITS2 sequences (data not shown).

Of the 6 case-patient–related properties from which environmental samples were taken (Table 2), 3 had a single sample that was culture positive for *S. schenckii* (clinical strain). Each of these 3 patients had purchased hay from the same commercial hay outlet (outlet 1). One of the hay samples from outlet 1 was also culturally positive for *S. schenckii* clinical group. These 4 isolates had identical ITS2 sequences and morphologic features that were similar to all our patient isolates available for testing (the patient 5 isolate was not available). *S. schenckii* (clinical strain) was not isolated from any of the control hay samples, although other members of the *Opiostoma–Sporothrix* complex were isolated in many environmental samples.

Table 2. Culture findings from environmental sampling

Hay sample source	No. samples	Samples culture positive for *Sporothrix schenckii** (GenBank accession no.)
Hay used by case-patient 2	2	0
Hay used by case-patient 4	2	1 (EF589128)
Hay used by case-patient 6	3	1 (EF589126)
Hay used by case-patient 7	4	0
Hay used by case-patient 8	3	1 (EF589125)
Hay used by case-patient 9	2	0
Hay outlet 1	5	1 (EF589127)
Hay outlet 2	4	0
Source of outlet 1	7	0
Control samples	18	0

**S. schenckii* clinical group as described in text.

Conclusions

The epidemiologic aspect of our study implicated contaminated hay as the source of an outbreak of sporotrichosis in the BMR region. Exposure was documented for 9 of the 11 case-patients. Most patients described contact with hay during gardening. Eight patients had contact with hay purchased at commercial suppliers in the Margaret River region.

Mycologic culture of hay samples confirmed that the hay was a possible source of infection. Half of the case-related properties tested were culture positive for the clinical strain of *S. schenckii*, as was a sample from a commercial hay supplier that had supplied hay to these properties. None of the control samples or samples from a farm that supplied hay to this hay supplier showed *S. schenckii* (clinical strain) on mycologic culture.

All Western Australia clinical isolates tested—including the BMR outbreak isolates, the isolates from the hay supplier, and 3 case-related hay samples—are indistinguishable by ITS2 sequencing and PFGE. These isolates differ from the environmental isolates of the *Ophiostoma–Sporothrix* complex tested from our survey and from eastern states' clinical isolates. Therefore, the epidemiologically implicated hay from the commercial hay suppliers was considered the likely source of the regional outbreak.

This finding prompted intervention in the community. Commercial hay suppliers cooperated by destroying any moldy hay on their properties and storing hay awaiting sale on rubber matting under cover. Information about the diagnosis and management of the infection was distributed to general practitioners in the area, and general information was distributed to the community through various sources such as community newspapers. Since the initial outbreak of sporotrichosis in the BMR region, the infection rate has decreased (Figure 2). Further distribution of the organism by hay suppliers across the region appears to have ceased with the introduction of infection control measures.

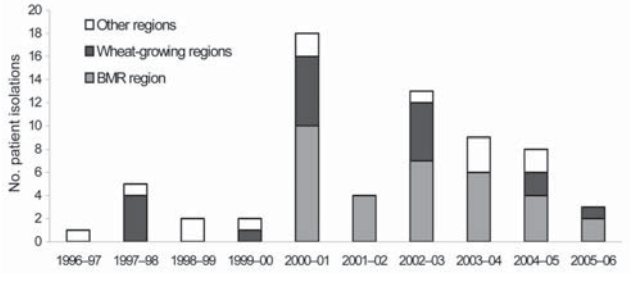

Figure 2. Sporotrichosis clinical isolation data from PathWest (QEII), July 1996–June 2006. BMR, Busselton-Margaret River.

Acknowledgments

We thank local environmental health officers for interviewing the local hay suppliers and investigating the suppliers' premises; the general practitioners for responding to surveillance requests on cases of sporotrichosis; and members of the PathWest Molecular Diagnostics Laboratory, including Brian Brestovac (now at Curtin University of Technology) and Gerry Harnett, for performing ITS sequencing.

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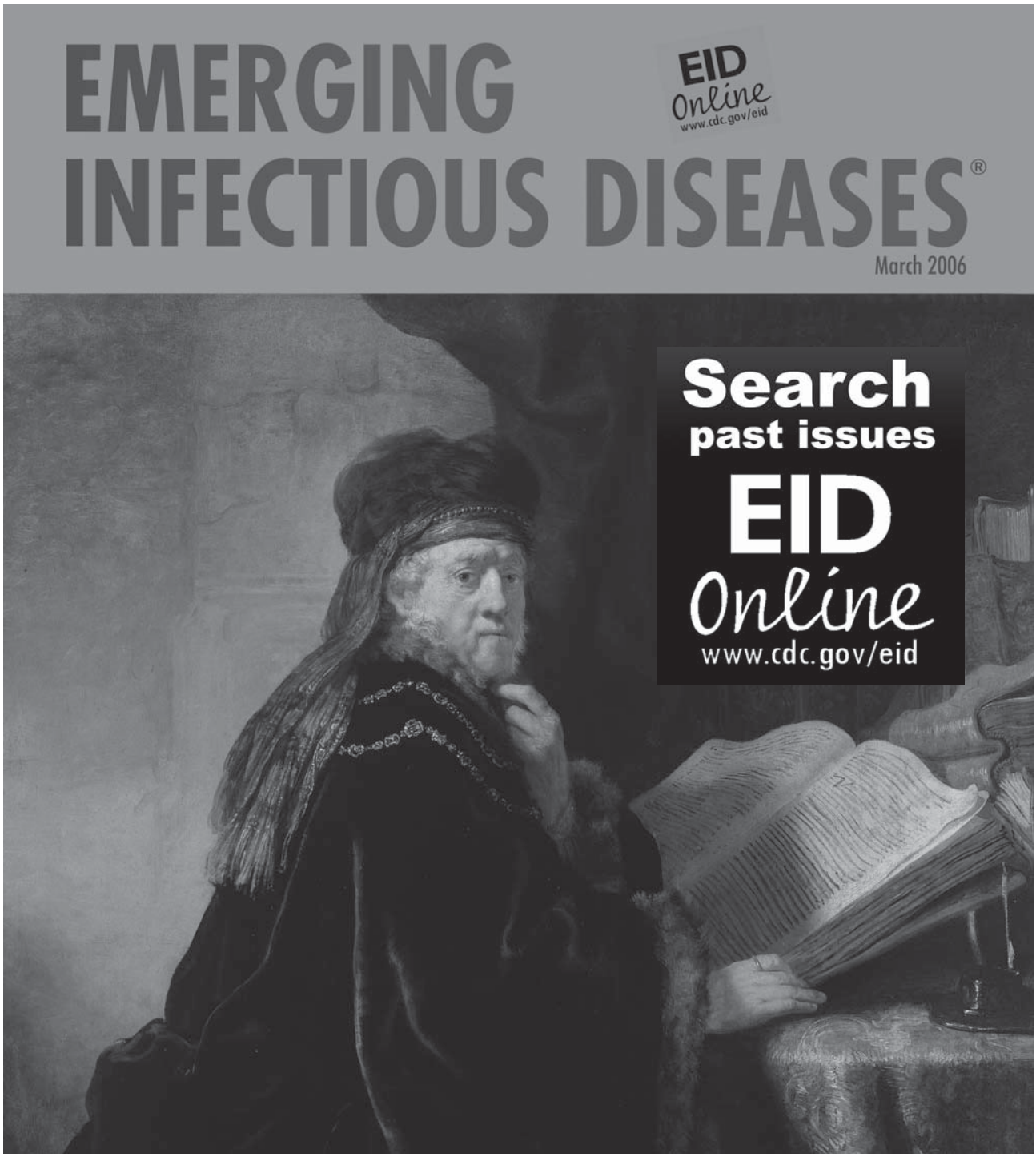

Rotavirus G5P[6] in Child with Diarrhea, Vietnam

Kamruddin Ahmed,* Dang Duc Anh,† and Osamu Nakagomi*

We detected rotavirus G5P[6] with a long RNA pattern in a Vietnamese child with diarrhea. Viral outer capsid protein VP7 and VP4 genes suggest that it likely originated from porcine rotavirus either by genetic reassortment or as whole virions. To our knowledge, this is the first report of human rotavirus G5 in Asia.

Rotaviruses cause 352,000–592,000 deaths per year in children <5 years of age (*1*). More than 80% of these deaths occur in developing countries (*1*). In multicenter trials, monovalent and pentavalent rotavirus vaccines were safe and highly efficacious for children in developing countries (*2*). Among developing countries in Asia, Vietnam is progressing rapidly toward introducing rotavirus vaccine (*3*). Rotavirus accounts for 55% of diarrheal diseases in Vietnam, prevalent serotypes are G1, G2, G3, G4, and G9, and 3% of rotaviruses were untypeable (*3*). This low percentage of untypeable strains was unanticipated because it is assumed that developing countries are the source of unusual rotavirus strains (*4*). Many people in Vietnam live in close contact with domestic animals, which may promote interspecies transmission of rotaviruses and reassortment of human and animal rotaviruses. Therefore, we hypothesized that in rural Vietnam, some rotavirus infections are caused by animal rotaviruses or animal–human reassortants.

The Study

We conducted a study by using double-stranded RNA obtained from 38 untypeable samples collected from children <5 years of age with rotavirus infections who were admitted to Khanh Hoa General Hospital and Ninh Hoa Hospital, Khanh Hoa Province, Vietnam. RNA was extracted from rotavirus-positive stool samples by using RNaid kits (MP Biomedicals/Qbiogene, Solon, OH, USA). Rotavirus-positive stool samples were detected by using Rotaclone ELISA kits (Meridian Bioscience. Inc., Cincinnati, OH, USA). Stool samples were collected from October 2003 through March 2004 as part of a rotavirus surveillance project conducted by 1 of the authors (D.D.A.) in collaboration with the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

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The G and P types were determined by using reverse transcription–PCR (*5*) Samples that could not be typed were subjected to nucleotide sequencing. Amplification of the nonstructural protein NSP4 gene was conducted as described (*6*).

Rotavirus genomic RNAs were visualized by polyacrylamide gel electrophoresis (*7*) and staining with a Silver Stain II kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) with a few modifications. The gel was incubated in staining solution for 45 min instead of 15 min, and in each washing step, the temperature of deionized water was maintained at 25°C.

VP7, the VP8* portion of outer capsid VP4, and NSP4 genes were sequenced by using the method previously described (*5*). Sequence similarity was searched with BLAST (www.ncbi.nlm.nih.gov/blast), multiple sequence alignment was conducted with ClustalW (www.ebi.ac.uk/clustalw), and phylogenetic trees were constructed by the neighbor-joining method using MEGA version 3.1 (*8*). Bootstrap analysis of 1,000 replicates was used to investigate branching of constructed trees. The *N*-glycosylation site of VP7 was predicted by using a NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/netnglyc).

A total of 38 samples were obtained (31 from Khanh Hoa General Hospital and 7 from Ninh Hoa Hospital). G and P types were determined for 35 samples. G type could not be determined for 3 samples (KH142, KH228, and KH210). However, these samples were typed as G2, G4, and G5, respectively, by a homology search of VP7 gene sequence. Thirty-three samples were typed as G1P[8], 2 as G2P[4], 1 as G4P[6], 1 as G5P[6], and 1 as G9P[8]. Three different electropherotypes with a long pattern were identified in 20 electrophoretic-positive samples. Of these, 18 samples showed the same electropherotype and all were typed as G1P[8]. Each of the G4P[6] and G5P[6] rotaviruses showed different electropherotypes. The sample that contained G5 rotavirus was isolated from a 7-month-old girl admitted to Khanh Hoa General Hospital in 2004 with diarrhea, fever, and malnutrition.

Sequence analyses of the VP7 gene showed that KH210 had more than 80% nucleotide and 90% amino acid identities with representative G5 rotaviruses from humans and pigs (Table 1). When we compared VP7 antigenic regions A (aa 87–101), B (aa 143–152), C (aa 208–223), and F (aa 235–242) (*9*) with representative strains of human G5 rotaviruses, unique substitutions were observed at aa positions 217 (V) of region C and 235 (Y) in region F. Other unique substitutions observed in KH210 were C, I, S, and N at positions 6, 29, 112, and 119, respectively.

Phylogenetic analysis of the amino acid sequence of the VP7 gene (Figure 1A) of G5 strains showed 2 genetically distinct groups; KH210 clustered with a porcine G5 strain from Australia. This group also contained a human

Table 1. Percentage identity of amino acid and nucleotide sequences of VP7 genes of KH210 and selected rotavirus G5 strains*

Strain	Species (country)	Deduced amino acid identity, %	Nucleotide identity, %
MRC 3105†	Human (Cameroon)	92	89
IAL-28	Human (Brazil)	91	85
CC117	Porcine (Argentina)	93	86
C134	Porcine (Argentina)	93	86
TFR-41	Porcine (Australia)	93	NA‡
JL94	Porcine (People's Republic of China)	91	85
OSU	Porcine (USA)	91	84
A34	Porcine (Venezuela)	92	84
A46	Porcine (Venezuela)	92	82
134/04–15	Porcine (Italy)	91	83
H1	Equine (USA)	91	85

*GenBank accession nos.: MRC3105/2000, AY327107; IAL-28, L79916; CC117, L35056; C134, L35058; TFR-41, P32547; JL94, AY538665; OSU, X04613; A34, L35059; A46, L35054; 134/04–15, DQ062572; H1, AF242393.
†Partial sequence.
‡NA, not available.

G5 strain from Cameroon. The human G5 strain from Brazil may belong to the other group. Similar to other G5 strains, VP7 of KH210 showed 1 potential *N*-glycosylation site at position 69.

The nucleotide and amino acid sequences of VP8* of strains KH210 and KH228 showed high identity (89%–93% and 90%–95%) with lineage I and relatively low identity (81%–86% and 85%–90%) with other lineages of P[6] (Table 2). Phylogenetic analysis of the amino acid sequence of VP8* of KH210, KH228, and other P[6] strains showed that Vietnamese strains clustered with Italian porcine strains of lineage Ic (Figure 1B).

Phylogenetic analysis of the amino acid sequence of NSP4 showed that KH210 was closely related to porcine

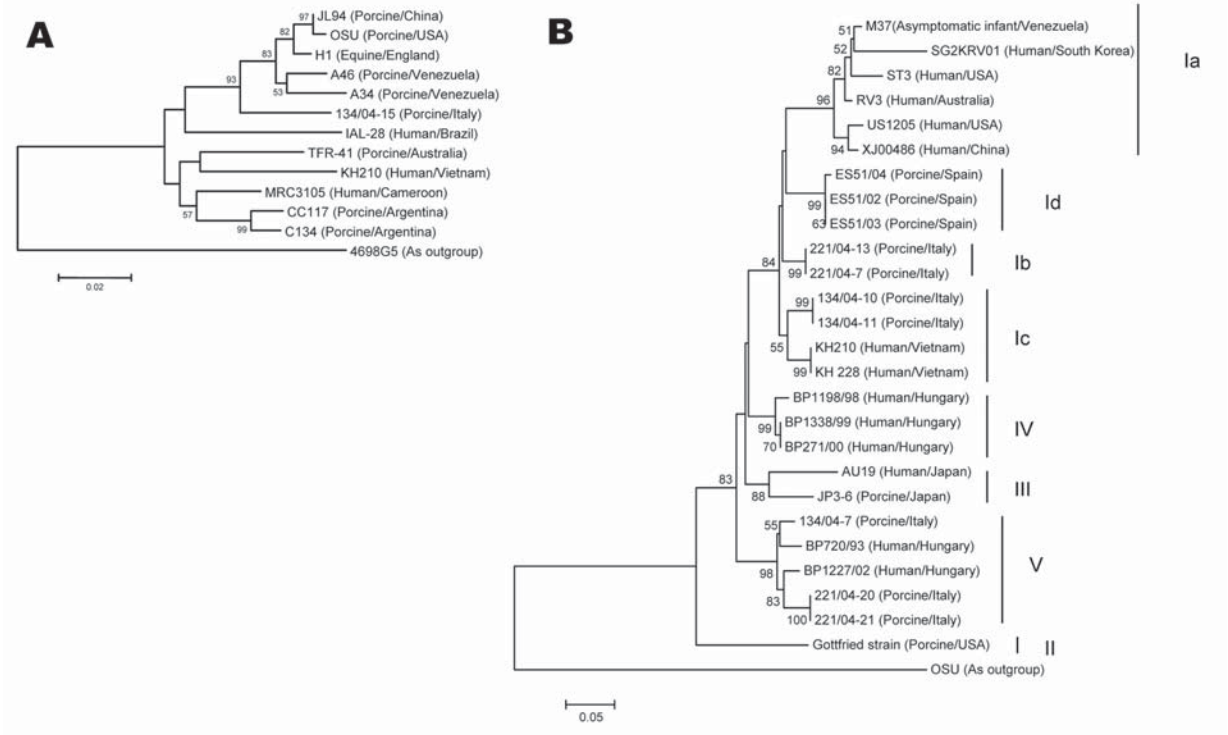

Figure 1. A) Phylogenetic tree constructed from deduced amino acid sequences of the VP7 gene of animal and human G5 rotaviruses. Strain 4695G5, an equine G3 strain, was used as an outgroup. Bootstrap values are expressed as percentages. Bootstrap value <50 is not shown. Strain KH210 clustered with the human G5 rotavirus from Cameroon (MRC3105) and other G5 rotaviruses of porcine origin from Australia and Argentina. The Brazilian human isolate of G5 rotavirus clustered with G5 rotaviruses of porcine and equine origin. Species of origin followed by country of isolation is shown in parentheses after the strain name. B) Phylogenetic tree constructed from the deduced amino acid sequences of the VP8* gene of rotaviruses representing all P[6] lineages. Strain OSU was used as an outgroup. Bootstrap values are expressed as percentages. A bootstrap value <50 is not shown. Strains KH210 and KH228 clustered with lineage Ic. Species of origin followed by country of isolation is shown in parentheses after the strain name. Scale bar shows genetic distance expressed as amino acid substitutions per site.

Table 2. Percentage identity of the partial amino acid and nucleotide sequences of VP8* genes of KH210 and selected rotavirus P[6] strains*

Strain (lineage)	Species (country)	Deduced amino acid identity, %	Nucleotide identity, %
M37 (Ia)	Human (Venezuela)	90	89
221/04–7 (Ib)	Porcine (Italy)	94	92
134/04–10 (Ic)	Porcine (Italy)	95	93
ES51/04 (Id)	Porcine (Spain)	92	91
Gottfried (II)	Porcine (USA)	85	82
AU19 (III)	Human (Japan)	87	81
BP1198/98 (IV)	Human (Hungary)	90	86
BP1227/02 (V)	Human (Hungary)	88	85

The amino acid and nucleotide sequence identity of VP8 genes of KH210 and KH228 were 99%. The amino acid sequence identity of VP8* genes between KH228 and Gottfried was 84%. Otherwise, the identity of amino acid and nucleotide sequences of VP8* genes of KH228 and selected rotavirus P[6] strains were same as for KH210. GenBank accession nos.: M37, L20877; 221/04–7, AY955303; 134/04–10, AY955299; ES51/04, AY955306; Gottfried, M33516; AU19, AB017917; BP1198/98, AJ621504; BP1227/02, AJ621505.

rotaviruses and belonged to genotype B (Figure 2), with identity of 94% to 96% to porcine stains and 91% to 95% to human strains of genotype B. Similar to the structure of most rotaviruses, 2 potential *N*-glycosylation sites were located at aa positions 8 and 18 (*8*).

Conclusions

G5 rotaviruses are isolated mainly from pigs. However, in 1994 these viruses were reported in samples from Brazilian children with diarrhea (*10*). Subsequently, G5 was identified as a cause of human infection in several states in Brazil (*11*), which suggests a broader distribution of this unusual serotype. Human G5 rotaviruses were then identified in children with acute diarrhea in Argentina (*12*) and Paraguay (*13*). Recently, human G5 rotavirus was reported in Cameroon (*4*), the first human G5 rotavirus isolated in Africa.

The human G5 rotavirus from Cameroon has a short RNA pattern. In contrast, the human G5 rotaviruses detected in Vietnam and Brazil have long RNA patterns. However, their VP7 genes are not in the same cluster in the phylogenetic tree. Brazilian G5 rotaviruses isolated from humans are usually found in combination with the P[8] genotype (*10*). This combination is a result of naturally occurring reassortment between human (P[8], Wa-like) and animal (G5, OSU-like) strains (*14*).

We have identified the combination of KH210 with genotype P[6]. This genotype is found predominantly in strains of porcine origin. VP8* of Vietnamese strains clustered with porcine strains isolated in Italy and belonged to the Ic lineage of P[6]. The NSP4 gene of KH210 belonged to genotype B and had high amino acid identities with both human and porcine rotaviruses of the same genotype. These findings are consistent with those of RNA-RNA hybridization (*7*) and sequence analysis (*15*). Human strain Wa and porcine strain OSU were shown to form 3 hybrid bands, including the one that corresponded to the NSP4 gene (*7*).

Rotaviruses from different species were shown by phylogenetic analysis to have similar NSP4 sequences that were clustered into the same NSP4 genotype. However, identifying the origin of the NSP4 gene in KH210 was not possible. On the basis of unique amino acid substitutions in VP7 and phylogenetic analysis of VP7 and VP8*, we believe that Vietnamese G5 strain most likely originated from porcine rotavirus by genetic reassortment or as whole virions. Although 2 strains with different G genotypes (G5

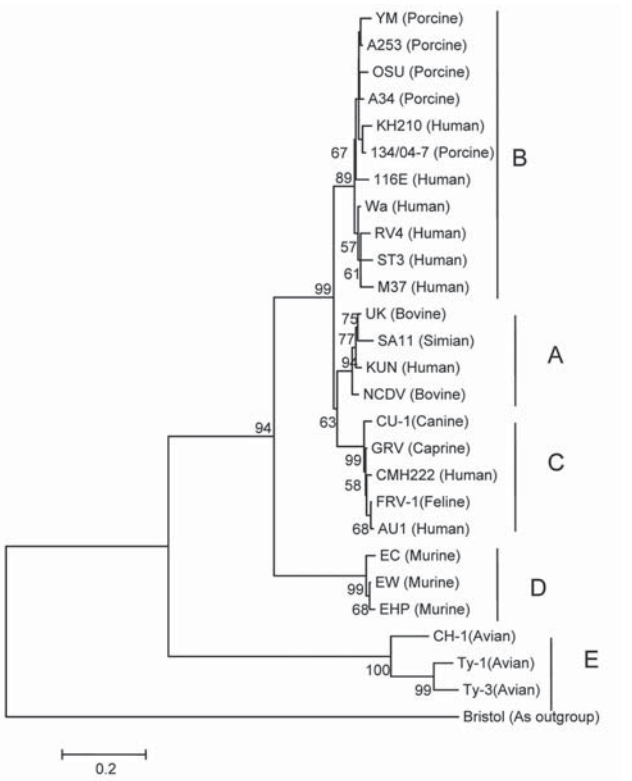

Figure 2. Phylogenetic tree constructed from the deduced amino acid sequences of the NSP4 gene of rotaviruses representing all genotypes. Strain Bristol, a group C rotavirus, was used as an outgroup. Bootstrap values are expressed as percentages. Bootstrap value <50 is not shown. Strain KH210 clustered with strains in genotype B. Species of origin is shown in parentheses after the strain name. Scale bar shows genetic distance expressed as amino acid substitutions per site.

for KH210 and G4 for KH228) have identical VP8* genes (Figure 1B), which suggests a contemporary genetic reassortment event, it is not known whether sequence divergence between KH210 and porcine rotaviruses indicates interspecies transmission.

More information will be obtained from sequencing of contemporary porcine rotavirus strains in Vietnam. Identification of a human G5P[6] strain in Vietnam will provide a rationale for expanded surveillance to understand its prevalence in Vietnam and other Asian countries.

Acknowledgments

The DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank accession nos. are AB257126 and AB303218 for the VP7 and NSP4 genes of strain KH210 and AB271687 and AB271688 for the VP8* gene of KH210 and KH228.

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Possible Autochthonous Malaria from Marseille to Minneapolis

Barbara Doudier,*† Hervé Bogreau,† Aaron DeVries,‡ Nicolas Ponçon,§ William M Stauffer III,‡ Didier Fontenille,§ Christophe Rogier,† and Philippe Parola*†

We report 2 cases of *Plasmodium falciparum* malaria in southern France in a French woman and an American man of Togolese origin who reported no recent travel to malaria-endemic countries. Both infections occurred after a stay near Marseille, which raises the possibility of autochthonous transmission. Entomologic and genotypic investigations are described.

Endemic malaria was eradicated from France during the middle of the past century. However, *Plasmodium falciparum* malaria has recently been reported in several Western European countries in persons with no history of travel or blood transfusion (*1*–*3*). The most recent case of an autochthonous case of vivax malaria was reported in 2006 in Corsica, where susceptible vectors persist (*4*). In southern France, a favorite destination for tourists, the last malaria cases in patients without any recent travel in malaria-endemic areas were reported in 1994 and 2000 (*5*,*6*). We report 2 cases that occurred in southern France during early spring and early summer 2006.

The Cases

On June 30, 2006, a 41-year-old woman was admitted to the North University Hospital in Marseille, France, with a 12-day history of fever, chills, and diarrhea. She had never traveled outside Europe and had no history of blood transfusion or injection drug use. She was born in Marseille and had lived there her entire life. Her home residence was >20 km from the nearest airport and 10 km from the seaport. The case-patient did not work outside the home and did not report any recreational activities near either location. Her neighborhood included families of Comorian descent. Laboratory testing demonstrated low leukocyte and erythrocyte counts and thrombocytopenia. Peripheral blood smear also demonstrated intra-erythrocytic forms consistent with *P. falciparum* infection with a parasitemia level of 0.1%. *P. falciparum* antigen was detected (Test NowICT; Fumouze, Levallois-Perret, France) and subsequently confirmed by PCR. Treatment comprised a 3-day regimen of quinine-clindamycin (*7)* and the case-patient recovered.

A 34-year-old man with tactile fever, bilateral frontal headache, and diarrhea was examined at a clinic in Minneapolis, Minnesota, on May 1, 2006. This man had lived in Togo, his birth country, all of his life until he emigrated to Minnesota in April 2000. Before becoming ill, he had traveled to Paris and from Paris by train to Marseille on April 7, 2006. Also during this timeframe, he reported a day trip to Camargue (Sorgues, France) where he sustained multiple mosquito bites. On April 24, 2006, he returned to Paris by train and then traveled by air a few hours later to Minneapolis. The first symptoms of malaria developed on April 27. His medical history included 4 episodes of malaria in childhood (none in the past decade) and no blood transfusions. On his last visit to Togo in December 2004, the case-patient had reported no chemoprophylaxis for malaria and had experienced no illness or intervening fevers since that time. Physical examination showed a moderately ill person with hematologic test results indicating thrombocytopenia and intra-erythrocytic ring forms consistent with *P. falciparum* malaria with a 3% parasitemia level. PCR testing confirmed *P. falciparum.* He was given 4 tablets of atovoquone-proguanil once a day for 3 days and recovered completely.

We conducted entomologic surveillance on July 26, 2006, in and around the first case-patient's residence in Marseille, 26 days after she became ill. No adult mosquitoes were found in the patient's home, on external staircases, in cellars located around the patient's home, or in surrounding buildings. Two pools of standing water were identified: a single pool (1 m^2, 2 cm deep) within a cellar in her building and another pool (10 m^2, 5 cm deep) located 20 m outside the building. No other containers or places that could collect water were identified. Several *Culex theileri* larvae were identified in the outdoor pool, but there was no evidence of *Anopheles* larvae.

DNA was extracted from blood samples by using the ENZA blood DNA kit according to the manufacturer's recommendations (Biofidal, Vaulx en Velin, France) and eluted in 100 μL of elution buffer per 250 μL of whole blood. Microsatellites loci (C4M79, Pf2689, TRAP, Pf2802, 7A11, and C4M69) were genotyped by fluorescent end-labeled PCR. Primers sequence, PCR conditions, and methods for genotyping have been described elsewhere (*8*,*9*). Drug-resistant mutations were genotyped. Genotyping of the 2 isolates demonstrated different alleles for the 6 microsatellite loci. We also observed different alleles from codon 59 (dihydrofolate reductase), 437 (dihydropteroate

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Table 1. Microsatellite loci genotyping and single nucleotide polymorphism in drug-resistant genes

	C4M79	Pf2689	Pf2802	7A11	C4M69	TRAP
Autochthonous 1*	190	87	141	88	378	132
Autochthonous 2†	221	95	138	99	364	149
3D7‡	221	87	138	94	364	137
W2‡	188	87	146	109	319	140

*Patient 1, *Plasmodium falciparum* diagnosed in France.
†Patient 2, *P. falciparum* diagnosed in the United States.
‡Two parasite strains (3D7 and W2) were genotyped as a positive control; water was used as a negative control.

synthase), and 76 (*P. falciparum* chloroquine-resistance transporter) (Tables 1, 2).

Conclusions

The World Health Organization defines introduced autochthonous malaria as that acquired by mosquito transmission from an imported case in an area where malaria is not a regular occurrence (*10*) In France, it is rare to find *P. falciparum* in a blood smear from patients who have not traveled to a malaria-endemic area in the previous 12 months. History of transfusion, organ transplantation, intravenous drug use, or mother-to-fetus transmission must also be excluded (*10*).

In Europe, some recent autochthonous malaria cases have been related to close proximity to airports and shipping ports receiving flights and water craft from malaria-endemic areas (*5*,*6*,*11*). Inadvertent carriage of infective *Anopheles* mosquitoes by airplane, ship, baggage, or bilge water may be responsible for these occurrences. Also, large populations of migrants from areas highly endemic for malaria (*12*) may act as human reservoirs for potential gametocyte carriers. Marseille has a large population of persons of Comorian origin, and most of the patients with imported malaria cases diagnosed in Marseille contracted the disease during a trip to the Comoros Islands (*13*,*14*). Our first case-patient's malaria may have been linked to her neighborhood, which included Comorian families who had recently traveled to Comoros. Despite the absence of *Anopheles* larvae near the first patient's neighborhood, local transmission cannot be excluded during the late spring and summer. Summer temperatures in Marseille induce a short *P. falciparum* sporogonic cycle (≈11 days at 28°C), which is compatible with mosquitoes' longevity at that time.

The Minnesota case-patient likely represents a second case of autochthonous malaria. First, this patient had no illness to suggest an untreated, active infection since his last trip to Togo. Second, even if he had been persistently infected with *P. falciparum* and was semi-immune, this case would still represent an extremely long incubation period (16 months). Third, onset of symptoms was acute, hematologic testing showed infection with a 3% parasitemia level, and the case-patient was moderately ill, which suggests a more recent exposure. Moreover, the genotype was monoallelic for each locus, indicating a clonal infection, which would be expected in areas of relatively low transmission frequency. In contrast, in areas of frequent endemic transmission, such as Togo, multiple allelic polymorphisms would be expected (*9*). Finally, genotyping demonstrated different clones of *P. falciparum*; this finding suggests potential multiple introductions, temporally related, of the parasite into the environment

The last malaria focus in continental France occurred near the end of World War II in the Camargue region, which was visited by our second case-patient in April 2006. However, large *Anopheles* populations, including potential vectors such as *An. hyrcanus*, *An. melanoon*, and *An. atroparus*, are still present from March to November (N. Ponçon and D. Fontenille, unpub.data) in southeastern France, generating an "anophelism without malaria" situation (*15*).

Migrants from malaria-endemic countries, climate, and *Anopheles* populations make southern France a favorable area for sporadic cases of autochthonous malaria in Europe. Given these 2 temporally related cases, clinicians should suspect malaria in patients with unexplained fevers who have recently traveled to areas of southern France.

Table 2. Two strains of *Plasmodium falciparum* obtained from 2 patients with no recent travel history in malaria-endemic areas*

	Dhfr					Dhps					Pfcrt
	51	16	108	164	59	613	540	581	436	437	76
Autochthonous 1†	–	GCA	AAC	ATA	TGT	GCC	AAA	GCG	TCT	GCT	ACA
Autochthonous 2‡	ATT	GCA	AAC	ATA	CGT	GCC	AAA	GCG	TCT	GGT	AAA
3D7§	AAT	GCA	AGC	ATA	TGT	GCC	AAA	GCG	TCT	GGT	AAA
W2§	ATT	GCA	AAC	ATA	CGT	TCC	AAA	GCG	TTT	GGT	ACA

*Dhfr, dihydrofolate reductase gene; Dhps, dihydropteroate synthase; Pfcrt, *P. falciparum* chloroquine-resistance transporter. Underlines indicate positions that can be mutated.
†Patient 1, *P. falciparum* diagnosed in France.
‡Patient 2, *P. falciparum* diagnosed in the United States.
§Two parasite strains (3D7 and W2) were genotyped as a positive control; water was used as a negative control.

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Waddlia chondrophila, a Potential Agent of Human Fetal Death

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We investigated the zoonotic potential of *Waddlia chondrophila*, a new *Chlamydia*-like abortigenic agent in ruminants. Anti-*Waddlia* antibody reactivity was tested by immunofluorescence and Western blot. *Waddlia* seroprevalence was higher in women who had had sporadic and recurrent miscarriages than in control women (p<0.001). *Waddlia* spp. may represent a cause of human fetal loss.

Approximately 15% of pregnancies end in miscarriage (*1*). However, a cause is identified in only 50% of these cases. Obligate intracellular bacteria, which fail to grow on media used routinely to isolate human pathogens, could represent yet unrecognized agents of miscarriage.

Chlamydia trachomatis, an intracellular bacterium, is the world's most common sexually transmitted bacterial pathogen (*2*). Because *C. trachomatis* is asymptomatic, most infected women remain untreated. Although the effect of *C. trachomatis* infection on pregnancy outcome is unclear, an increased prevalence of *C. trachomatis* immunoglobulin (Ig) G antibodies in women with a history of miscarriage has been observed (*2*,*3*). *Chlamydophila abortus* is the most common etiology of abortion in ruminants (*4*) and can also cause miscarriage in pregnant women exposed to infected animals.

Waddlia chondrophila, another *Chlamydiales*, is a new abortigenic agent in bovines (*5*,*6*). This obligate intracellular bacterium was isolated from aborted fetuses in the United States (*7*) and in Germany (*8*). A serologic study further supported the abortigenic role of *Waddlia* in bovine species (*6*). Moreover, infection of bovine fetuses with *Waddlia* was associated with their deaths within 2 weeks (*6*). In this study, we tested women with sporadic and recurrent miscarriages for antibody reactivity against *Waddlia* and compared seroprevalence with that found in a control group of women with uneventful term pregnancies.

The Study

From July 2004 to March 2005, we studied 69 women with sporadic miscarriages (SM), 200 women who had suffered from recurrent miscarriages (RM), and 169 control women who had had uneventful pregnancies (Table 1). The RM group comprised women who had ≥3 miscarriages (*1*) and who had attended the Recurrent Miscarriage Clinic of St Mary's Hospital, London, the largest specialist referral center in Europe.

Immunofluorescence tests were performed (*9*); we used *W. chondrophila* strain ATCC VR-1470 as antigen and we screened sera at a 1:64 dilution with FluolineH (bioMerieux, Marcy l'Etoile, France). Antigen was isolated as described (*10*,*11*). Mice and rabbit anti-*Waddlia* antibodies were used as positive controls with a fluorescein-conjugated anti-mouse and anti-rabbit globulin. Sera that exhibited an Ig titer ≥64 were tested for IgG and IgM reactivity by using corresponding anti-human Ig fluorescein (FluolineG or FluolineM, bioMérieux) and serial 2-fold dilutions of serum. IgG and IgM positivity cut-offs were ≥1:64 and ≥1:32, as proposed for other chlamydia-like organisms (*4*). One hundred women had an anti-*Waddlia* IgG titer ≥64 (Table 1). Seroprevalence was higher for patients who had sporadic (31.9%) and recurrent (33%) miscarriages than that for women who had had uneventful pregnancies (7.1%, p<0.001 when comparing either SM or RM groups to controls). One woman had a positive IgM titer of 64 and an IgG titer of 512.

To confirm the specificity of immunofluorescence, we performed Western blot analyses on all *Waddlia*-positive sera samples. Western blot was performed (*9*), but *Waddlia* was used as the antigen. A polyclonal peroxidase-labeled anti-human IgG (Dako, Glostrup, Denmark) was used as a secondary antibody. The presence of anti-*Waddlia* IgG antibodies was confirmed by Western blot in 97 of the 100 positive samples by immunofluorescence (Table 1; Figure 1, panels B and C). By using *Waddlia* spp. hyperimmune mouse and rabbit sera and corresponding peroxidase-conjugated anti-mouse/rabbit sera, we obtained similar patterns of 61-, 55-, 53-, 45-, 41-, 38- and 30-kDa proteins (Figure 1, panels D and E). Antibody reactivity against the 55-, 53-, 45-, 41- and 38-kDa proteins disappeared after adsorption with 10^8 *Waddlia* antigen for 48 hours, which demonstrated the specificity of the antibody response (Figure 1, panel F).

For further statistical analyses, only patients whose samples were positive for *Waddlia* spp. by immunofluorescence and confirmed by Western blot were considered seropositive for *Waddlia* spp. (n = 97). In all age groups, the rate of *Waddlia* seropositivity was higher in patients who had miscarried than in those who had not (data not shown). Moreover, most women with anti-*Waddlia* antibodies did not exhibit serologic reactivity against *C. trachomatis* (Table 2).

In a multivariate logistic regression adjusted for age, ethnicity, contact with animals and *C. trachomatis* serostatus (Figure 2), miscarriage (SM/RM) remained strongly

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Table 1. Characteristics of study patients according to miscarriage history*

Characteristics	Controls (n = 169), no. (%)	Sporadic miscarriages (n = 69), no. (%, p value†)	Recurrent miscarriages (n = 200), no. (%, p value†)
Age, y			
Median (IQR, p value†)	30.4 (25–35)	31.7 (27–36, 0.18)	35.4 (31–39, <0.001)
No. pregnancies			
1	90 (53.3)	32 (46.4)	0
2	46 (27.2)	19 (27.5, 0.67‡)	0 (0, <0.001‡)
>2	33 (19.5)	18 (26.1)	200 (100)
Mean (SD, p value†)	1.8 (1.1)	2.1 (1.5, 0.23)	5.2 (3, <0.001)
Parity			
0	0 (0)	49 (71)	113 (56.5)
1	115 (68.1)	11 (15.9, <0.001‡)	59 (29.5, <0.001‡)
2	31 (18.3)	4 (5.8)	19 (9.5)
>2	23 (13.6)	5 (7.3)	9 (4.5)
Mean (SD, p value†)	1.54 (0.95)	0.52 (1.01, <0.001)	0.64 (0.9, <0.001)
Miscarriages			
Early (≤12 weeks)	0	52 (75.4, <0.001)	196 (98, <0.001)
Late (>12 weeks)	0	13 (18.9, <0.001)	51 (25.5, <0.001)
Stillbirth (>24 weeks)	0	4 (5.8, <0.001)	11 (5.5, 0.002)
Alive child	169 (100)	21 (30.4, <0.001)	78 (39, <0.001)
Ethnicity			
White	80 (47.3)	34 (49.3, 0.79)	132 (66, <0.001)
Black	35 (20.7)	10 (14.5, 0.27)	22 (11, 0.01)
Asian	31 (18.3)	18 (26.1, 0.18)	34 (17, 0.74)
Other	22 (13)	6 (8.7, 0.35)	8 (4, 0.002)
Contact with animals	29 (17.2)	11 (15.9, 0.82)	70 (35, <0.001)
Cat	18 (10.7)	5 (7.3, 0.42)	37 (18.5, 0.035)
Dog	15 (8.9)	6 (8.7, 0.97)	28 (14, 0.13)
Fish	1 (0.6)	0 (0, 1)	8 (4, 0.043)
Rodents	0	2 (2.9, 0.08)	7 (3.5, 0.017)
Other	3 (1.8)	0 (0, 0.63)	12 (6, 0.041)
Chlamydia trachomatis (IgG titer>50)	15 (8.9)	9 (13, 0.33)	39 (19.5, 0.004)
Positive serology for *Waddlia* spp.			
IgG titer >64	12 (7.1)	22 (31.9, <0.001)	66 (33, <0.001)
IgG titer >128	6 (3.6)	10 (14.5, 0.002)	29 (14.5, <0.001)
Western blot *Waddlia* IgG			
≤1 band	12 (7.1)	22 (31.9, <0.001)	63 (31.5, <0.001)
≤2 bands	6 (3.6)	15 (21.7, <0.001)	51 (25.5, <0.001)
≤3 bands	3 (1.8)	9 (13, <0.001)	28 (14, <0.001)
≤2 specific bands§	1 (0.6)	9 (13, <0.001)	22 (11, <0.001)

*Controls, women with uneventful pregnancies; IQR, interquartile range.
†Compared with control group.
‡p value for ordered categories (Pearson χ^2 test).
§According to *Waddlia* hyperimmune mouse serum, bands at 61-, 55-, 53-, 45-, 41-, 38-, and 30-kDa are considered specific for *Waddlia* spp.

associated with *Waddlia* seropositivity (odds ratio [OR] 4.9, 95% confidence interval [CI] 2.5–9.4). In this model, miscarriage was also independently associated with age (OR 2.9, 95% CI 2.0–4.1) and *C. trachomatis* seropositivity (OR 2.3, 95% CI 1.2–4.5). Additional multivariate models confirmed the association between *Waddlia* IgG seropositivity and miscarriage, with ORs ranging from 4.9 to 6.2.

Noninfectious causes of miscarriage have also been recorded (Table 2). When studying only the 322 patients without known concurrent conditions, the strong correlation between *Waddlia* seropositivity and miscarriage was still present (68/195 [34.8%] of patients who had had a miscarriage vs. 10/127 [7.9%] of patients who had not miscarried had anti-*Waddlia* antibodies; p<0.001).

Waddlia seropositivity was associated with early miscarriage (p<0.001, Table 2). No difference in ethnicity was observed between those who were IgG positive for *Waddlia* spp. and those who were negative. Moreover, there was no difference in *C. trachomatis*, *Chlamydophila pneumoniae*, or *Cp. psittaci* seropositivity between women who had anti-*Waddlia* antibodies and those who did not, which suggests that *Waddlia* antibodies do not cross-react with *Chlamydiaceae*. Moreover, only 7 (1.6%) of 438 patients had a *Parachlamydia* IgG titer ≥64. With such a low

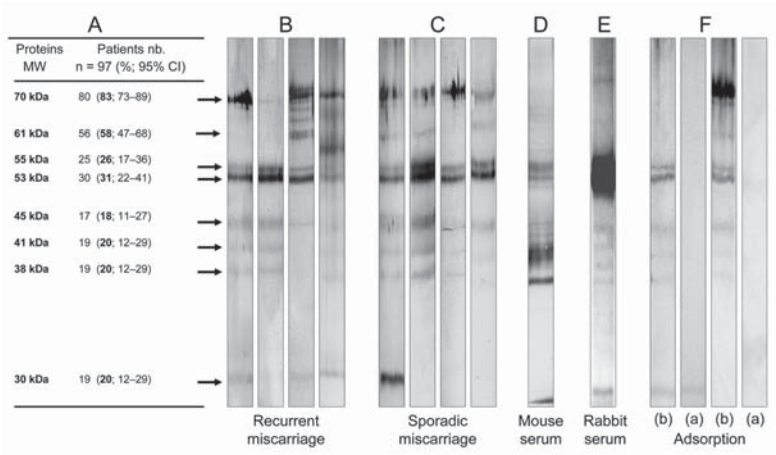

Figure 1. Western blot analyses. A) Molecular weight and frequency of IgG reactivity of *Waddlia* proteins, as determined by Western blots. B) and C) Four representative Western blot patterns of *Waddlia* IgG positive sera from recurrent and sporadic miscarriage groups. D) and E) Representative Western blot pattern of positive control (*Waddlia* hyper-immune mouse and rabbit serum, respectively). F) Western blot performed with *Waddlia* IgG positive sera, taken from patients who had miscarried before (b) and after (a) adsorption with *Waddlia* antigen. MW, molecular weight; nb, number.

Table 2. Characteristics of patients in the study according to their *Waddlia* serostatus

	Waddlia negative* (n = 341)	*Waddlia* positive† (n = 97)	p value
Age, y			
Median (interquartile range)	33 (28–37)	36 (31–39)	<0.001
No. pregnancies			
1	107 (31.4)	15 (15.5)	
2	62 (18.2)	16 (16.5)	0.006‡
>2	172 (50.4)	66 (68)	
Mean (SD)	3.2 (2.6)	4 (3.1)	0.005
Parity			
0	108 (31.7)	54 (55.7)	
1	156 (45.8)	29 (29.9)	0.001‡
2	49 (14.4)	5 (5.2)	
>2	28 (8.2)	9 (9.3)	
Mean (SD)	1.04 (1.04)	0.7 (1)	<0.001
Miscarriages			
Early (≤12 wk)	170 (49.9)	81 (83.5)	<0.001
Late (>12 wk)	47 (13.8)	17 (17.5)	0.36
Stillbirth (>24 wk)	12 (3.5)	3 (3.1)	1
Alive child	228 (66.9)	40 (41.2)	<0.001
Ethnicity			
White	184 (54)	62 (63.9)	0.08
Black	52 (15.3)	15 (15.5)	0.96
Asian	70 (20.5)	13 (13.4)	0.11
Other	29 (8.5)	7 (7.2)	0.68
Contact with animals	76 (22.3)	34 (35.1)	0.011
Cat	42 (12.3)	18 (18.6)	0.12
Dog	35 (10.3)	14 (14.4)	0.25
Fish	6 (1.8)	3 (3.1)	0.42
Rodent	7 (2.1)	2 (2.1)	1
Other	12 (3.5)	3 (3.1)	1
Noninfectious miscarriage causes	97 (28.5)	19 (19.6)	0.08
Autoimmune disease	19 (5.6)	1 (1)	0.09
Hypertensive disorder	8 (2.4)	3 (3.1)	0.71
Endocrine pathology	13 (3.8)	2 (2.1)	0.54
Anatomic abnormalities	4 (1.2)	2 (2.1)	0.62
Additional serologies (titers)			
Chlamydia trachomatis (IgG≥50)	49 (14.7)	14 (14.3)	0.98
Clamydophila pneumoniae (IgG≥64)	161 (47.2)	38 (39.2)	0.16
Cp. psittaci (IgG≥1/64)	20 (5.9)	6 (6.2)	1

*Patients with a *Waddlia* immunoglobulin G (IgG) titer <64 (n = 338) or not confirmed by Western blot analysis (n = 3).
†Patients with a *Waddlia* IgG titer >64 and confirmed by Western blot analysis.
‡p value for ordered categories (Pearson χ^2 test).

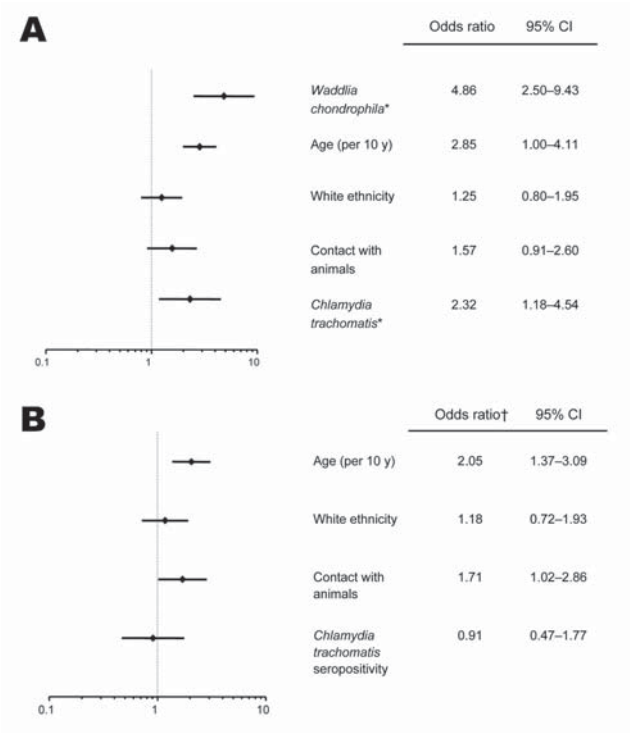

Figure 2. Multivariate analyses. A) Multivariate analysis adjusted for all variables listed in this figure and showing the independent association of age, positive *Waddlia* serologic results and positive *Chlamydia trachomatis* serologic results for women who had had a miscarriage. B) Multivariate analysis adjusted for all variables listed in this figure and showing the independent association of animal contact and advancing age with serologic evidence of *Waddlia* infection. *, seropositivity; †, odds ratio for *Waddlia* seropositivity.

Parachlamydia prevalence, cross-reactivity with this chlamydia-like organism is unlikely to explain the high *Waddlia* seroprevalence observed in the miscarriage groups.

Women who were IgG seropositive for *Waddlia* spp. were more likely to have had contact with animals. In a multivariate logistic regression model adjusted for age, ethnicity, and *C. trachomatis* serostatus, those who had had previous contact with animals were more likely to exhibit anti-*Waddlia* antibodies (OR 1.7, 95% CI 1.0–2.9, Figure 2). In this model, *Waddlia* IgG–positive serologic test results were also independently associated with age (OR 2.1, 95% CI 1.4–3.1).

Conclusions

This study demonstrates a strong association between the presence of *W. chondrophila*–specific IgG antibodies and early fetal loss. Cross-reactivity with other microorganisms seems an unlikely explanation for our results because *W. chondrophila* did not react with monoclonal or polyclonal antisera directed against *Rickettsia*, *Coxiella*, *Wolbachia*, *Anaplasma*, and *Chlamydia* spp. (*8*,*12*). We did not detect any cross-reactivity of *W. chondrophila* with *C. trachomatis*, *Cp. pneumoniae*, and *Cp. psittaci*. Moreover, the molecular weights of Waddlial immunoreactive proteins obtained by Western blot are clearly different than those reported for *C. trachomatis* or *Cp. pneumoniae* (*13*).

With the exception of 1 patient who had IgM, only IgG antibody reactivity against *W. chondrophila* was observed. Because IgG antibodies may persist for years after an acute infection has resolved (*3*), the underlying mechanism of miscarriage due to *W. chondrophila* may involve reactivation of a latent asymptomatic waddlial infection, endometrial damage from a past waddlial infection, or an immune response to an epitope shared by a waddlial and fetal antigen, as proposed for *C. trachomatis* (*3*).

The association we found between contact with animals and positive serologic results for *Waddlia* spp. raises the zoonotic potential of this bacterium. This hypothesis is further supported by the range of hosts for *Waddliaceae* (*4*). Other modes of transmission are possible (e.g., contaminated water) because free-living amebae may serve as hosts for *Waddlia* spp. (*14*) and are widespread in water networks (*15*). *Waddlia* spp. may also be transmitted through ingestion of contaminated cow milk. Finally, *Waddlia* spp. might represent a sexually transmitted disease.

Further investigations are urgently needed to define how *Waddlia* spp. infection may be acquired. To confirm the role of *W. chondrophila* in miscarriage, it will be important to isolate this intracellular bacterium from miscarriage products or confirm its presence in the placenta by immunohistochemistry or PCR. This may be difficult to achieve, if, as suggested for *C. trachomatis (3)*, *W. chondrophila* causes miscarriage indirectly, e.g., through increased cytokine production or molecular mimicry with fetal antigens. To our knowledge, this work provides the first evidence that *W. chondrophila* may be implicated in human fetal death.

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Norovirus Detection and Genotyping for Children with Gastroenteritis, Brazil

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During 1998–2005, we analyzed stool samples from 289 children in Rio de Janeiro to detect and genotype norovirus strains. Previous tests showed all samples to be negative for rotavirus and adenovirus. Of 42 (14.5%) norovirus-positive specimens, 20 (47.6%) were identified as genogroup GI and 22 (52.3%) as GII.

Noroviruses, a genus within the family *Caliciviridae*, have emerged as an important cause of epidemic and sporadic diarrheal disease in humans of all ages worldwide (*1–3*). The norovirus genome consists of a single strand of positive-sense RNA organized into 3 open reading frames (ORFs). ORF1 encodes nonstructural proteins such as RNA-dependent RNA polymerase, ORF2 encodes viral capsid protein 1, and ORF3 encodes a small capsid protein (viral capsid protein 2) associated with stability of viral capsid protein 1 (*4–6*). According to nucleotide sequence analysis of the polymerase and capsid regions, noroviruses are classified into 5 genogroups, GI to GV; each genogroup can be further divided into several clusters or genotypes. Genogroups GI, GII, and GIV have been found in humans, though GII seems to be the predominant strain around the world (*4,7–11*). To detect and genotype norovirus in stool samples from Brazilian children ≤10 years of age who had acute diarrhea, we used real-time Light Cycler reverse transcription–PCR (RT-PCR) and conventional RT-PCR assays.

The Study

From January 1998 through May 2005, a total of 2,421 fecal specimens were collected from children ≤10 years of age (median age 2.3 years) with acute diarrhea in Rio de Janeiro, Brazil. Of these, 478 (19.7%) specimens were collected from hospitalized children (inpatients) and 1,943 from outpatient children (341 [14.1%] from the emergency department and 1,602 [66.2%] from the walk-in clinic). Of these samples, 14.3% were positive for rotavirus (9.8%) or adenovirus (4.5%). The median age was 12.5 months for rotavirus-positive patients and 12.2 months for adenovirus-positive patients. Overall, of the hospitalized, emergency department, and walk-in clinic patients, 11.7%, 6.2%, and 10.0%, respectively, had samples positive for rotavirus, and 4.8%, 4.1%, and 4.4%, respectively, had samples positive for adenovirus. Enteropathogenic bacteria such as *Escherichia coli*, *Salmonella* spp., *Yersinia enterocolitica*, *Campylobacter* spp., and *Shigella* spp. were found in 8% of the specimens. Seven mixed infections were detected (2 adenovirus and *Salmonella* spp., 2 adenovirus and *E. coli*, 1 adenovirus and *Campylobacter* spp., 1 rotavirus and *Salmonella* spp., and 1 rotavirus and *E. coli*).

We selected 289 specimens that represented a random subset of samples that had prior negative results for rotavirus and enteric adenovirus. Of these 289, 117 were collected from inpatients and 172 from outpatients (89 emergency department and 83 walk-in clinic). The mean and the median age of the tested patients was 3.1 years. Suspensions of stool (10%) were prepared in diethylpyrocarbonate-treated water and Vertrel XF (Miller-Stephenson, Sylmar, CA, USA) and clarified by centrifugation at 2,100× *g* for 10 min. We used 200 μL of suspension for RNA extraction with the NucliSens extraction kit (Organon Tekninka, Durham, NC, USA) according to the manufacturer's instructions. The RNA was eluted in 50 μL of elution buffer and stored at –70°C until use.

A total of 240 samples were tested for norovirus RNA by Light Cycler PCR that used primers and probes for ORF1/ORF2 junction region specific for norovirus GI and GII, as described (3,*12*), and by the Light Cycler RNA Amplification Kit Hybridization Probes (Roche, Basel, Switzerland). Samples that showed a positive threshold at <38 cycles were considered positive. The 49 remaining samples were tested only by conventional RT-PCR, as described (*5*). Conventional RT-PCR was performed with the QIAGEN OneStep RT-PCR Kit (QIAGEN, Valencia, CA, USA). The RNA samples were subjected to 1 cycle of reverse transcription (42°C, 10 min) followed by 5 min at 95°C. PCR was performed for 40 cycles, each consisting of 1 min at 94°C, 1 min at 40°C (for GI detection) or 1 min at 44°C for (for GII detection), 1 min at 72°C, and a final extension cycle of 10 min at 72°C.

We selected 6 samples that were positive by real-time Light Cycler PCR (2 GI and 4 GII) for analysis by conven-

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tional RT-PCR with specific primers in capsid region D of norovirus GI and GII, as described above. The amplified cDNA samples were purified from the gel by using QIAquick gel extraction kit (QIAGEN), and the sequences were determined with the BigDye terminator cycle sequencing kit and the ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) by using the same primers as used for the conventional RT-PCR. The nucleotide sequences of the amplicons were aligned with corresponding sequences of selected norovirus strains available in the GenBank database and analyzed by using the CLUSTAL V algorithm of the MegAlign program in the DNASTAR software package (Madison, WI, USA). The nucleotide sequences obtained in this study were deposited in GenBank under accession nos. DQ496212, DQ496213, DQ496214, DQ496215, DQ496216, and DQ496217.

Of the 289 fecal specimens tested, 42 (14.5%) were positive for norovirus: 36 (15%; n = 240) by real time Light Cycler PCR and 6 (12.2%; n = 49) by conventional RT-PCR. These percentages correspond only to single infections because we did not test samples already known to be positive for other pathogens such as rotavirus and adenovirus. Positive samples and genogroups varied by year with no obvious yearly pattern (Table 1). Although norovirus is often referred to as "the winter vomiting disease," we detected infection throughout the year, with no seasonal pattern (Figure). Norovirus infections were equally common among outpatients and inpatients. Among 117 inpatients, 18 (15.4%) had positive norovirus test results compared with 24 (14%) of 172 outpatients (11 emergency department and 13 walk-in clinic). Although the disease caused by norovirus is described as mild (diarrhea, vomiting, abdominal pain, and fever) and generally does not lead to hospitalization (*13*,*14*), of 42 norovirus-infected children, 29 (69%) were either hospitalized or received medical care in the emergency department, which suggests that they had a more severe illness. Only 13 (31%) of the 42 norovirus-infected children attended the walk-in clinic, which suggests that they had mild disease (Table 2). Other than diarrhea, fever was the most common symptom among the

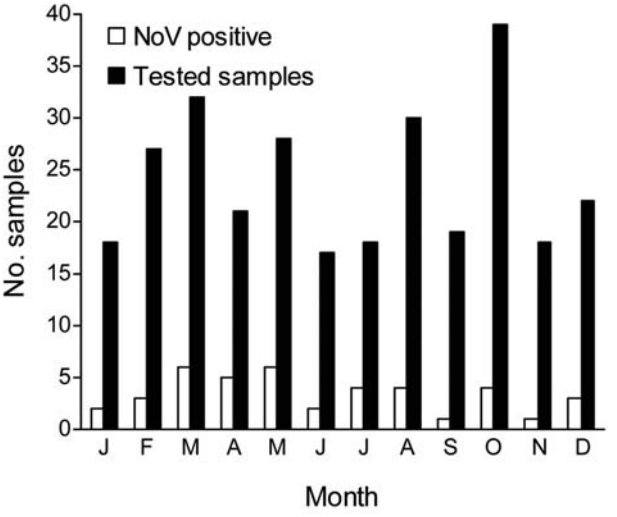

Figure. Seasonal distribution of norovirus (NoV) infections in Rio de Janeiro, Brazil, 1998–2005.

42 norovirus-positive patients in this study, reported for 11 (26.2%) patients. Vomiting only was described for 8 (19.0%); vomiting and fever was described for 6 (14.3%). No mixed infection with bacteria was observed.

Although norovirus belonging to genogroup GII is considered the most prevalent strain worldwide (*7*–*9*,*11*,*15*), we found no important difference in the prevalence of the 2 genogroups detected in our study. Overall, 20 (48%) of the 42 samples were identified as genogroup GI and 22 (52%) as GII (Table 1). No statistically significant difference in the prevalence of GI and GII was observed between inpatients and outpatients.

Conclusions

Our study documents that noroviruses are a common cause of acute gastroenteritis in children who are inpatients or outpatients in Brazil and are likely second only to rotavirus as a cause of severe childhood diarrhea. Our study was exploratory and has limitations. Nonetheless, it documents how common norovirus infections may be and indi-

Table 1. Distribution of norovirus-positive samples detected in Rio de Janeiro, Brazil, 1998–2005*

Year	Real-time reverse transcription–PCR		Conventional reverse transcription–PCR		Total no. positive samples/genogroup
	No. samples tested	No. positive samples/genogroup	No. samples tested	No. positive samples/genogroup	
1998	23	4/3GI + 1GII	0	NA	4/3GI + 1GII
1999	29	3/GII	5	1/GI	4/1GI + 3GII
2000	31	4/2GI + 2GII	7	0	4/2GI + 2GII
2001	26	4/GII	0	NA	4/GII
2002	31	5/4GI + 1GII	10	0	5/4GI + 1GII
2003	32	8/5GI + 3GII	9	1/GI	9/6GI + 3GII
2004	39	4/1GI + 3GII	16	3/GI	7/4GI + 3GII
2005	29	4/GII	2	1/GII	5/5GII
Total	240	36/15GI + 21GII	49	6/5GI + 1GII	42/20GI + 22GII

*NA, not applicable.

Table 2. Distribution of all tested samples by age groups and patient status, Rio de Janeiro, Brazil, 1998–2005

	Outpatients			Inpatients		
Age, y	No. tested	PCR-positive, no. (%)	PCR-negative, no. (%)	No. tested	PCR-positive, no. (%)	PCR-negative, no. (%)
<1	29	4 (13.8)	25 (86.2)	45	5 (11.1)	40 (88.9)
1–5	64	9 (14.0)	55 (86.0)	88	15 (17.0)	73 (83.0)
6–10	24	5 (20.8)	19 (79.2)	39	4 (10.3)	35 (89.7)

cates that further study will be necessary to assess their role among Brazilian children, to understand the epidemiology of the disease, and to seek evidence of immunity in children, which might encourage development of a vaccine.

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Atypical Q Fever in US Soldiers

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Q fever is an emerging infectious disease among US soldiers serving in Iraq. Three patients have had atypical manifestations, including 2 patients with acute cholecystitis and 1 patient with acute respiratory distress syndrome. Providers must be aware of Q fever's signs and symptoms to avoid delays in treatment.

Q fever, caused by infection with *Coxiella burnettii,* is an emerging infectious disease among US soldiers deployed to Iraq and Afghanistan; >30 cases have been reported (*1*–*3*). We describe 3 cases of Q fever in soldiers treated from July through December 2006 at Walter Reed Army Medical Center (WRAMC).

The Patients

In December 2006, 1 week after returning from Iraq, a 22-year-old white male Army National Guard member was seen at a New Hampshire hospital, with flulike symptoms, pleuritic chest pain, and mild abdominal pain. His initial examination noted temperature of 38.3°C, leukocytes 3.3×10^9 cells/μL (normal 4.5–10.5 × 10^3 cells/μL), platelets 121×10^3 cells/μL (normal 150–450 × 10^3 cells/μL), aspartate aminotransferase (AST) 144 IU/L (normal 15–46 IU/L), and alanine aminotransferase (ALT) 154 IU/L (reference 11–66 IU/L). He was admitted and treated with ceftriaxone and azithromycin. Although his fever decreased within 48 h, he had persistent abdominal pain, worsening liver function test results (AST 779, ALT 993, alkaline phosphatase 269 U/L [reference 38–126 U/L]), and increasing shortness of breath. An ultrasound examination of the right upper quadrant showed hepatosplenomegaly and a thickened gall bladder wall without evidence of cholelithiasis. Despite initially normal chest radiographic results, a repeat radiographic examination showed bilateral pulmonary infiltrates. Ceftriaxone therapy was discontinued, pipercillin/tazobactam therapy was started, and azithromycin was continued. General surgery stated that the patient had a nonsurgical abdomen. After consultation with WRAMC, the patient was given a dose of doxycycline and gentamicin before being transferred to a New Hampshire medical center. Blood cultures and serologic tests for Epstein Barr virus and cytomegalovirus were pending. A computed tomographic (CT) examination of the chest, abdomen, and pelvis (Figure, left panel) showed gall bladder wall thickening (10 mm) without ductal dilatation, hepatosplenomegaly, and bilateral ground glass pulmonary infiltrates. Serologic tests were negative for hepatitis B and C. Thick and thin smears were negative for parasitic disease. Despite the findings on CT scan, the patient began to improve clinically and had resolution of abdominal pain and shortness of breath. He was transferred to WRAMC, where he continued to improve. Pipercillin/tazobactam was discontinued, but doxycycline was continued. A presumptive diagnosis of Q fever was made, and he was discharged to complete a 14-day course of doxycycline. Serologic tests for *C. burnettii* were positive with a phase 2 immunoglobulin M (IgM) titer of 256 (negative <1:64), phase 2 IgG titer of 128 (negative <16), and negative phase 1 serologic results. A month later he felt well and had normal liver function test results. No exposure factors were identified.

The second case occurred in December 2006, when a previously healthy 24-year-old male Army National Guard member was admitted to the 28th Combat Support Hospital (CSH) in Baghdad, Iraq, with flulike symptoms, mild nausea, and a dry, 10-day cough. At admission, his temperature was 40.2°C, but his other vital signs were normal. He had mild epigastric tenderness to palpation; otherwise, examination results were normal. Laboratory results included leukocytes 3.9×10^3 cells/μL, platelets of 130×10^3 cells/μL, alkaline phosphatase 104 U/L, AST 824 U/L, ALT 786 U/L, total bilirubin 1.2 mg/dL (reference 0.2–1.3 mg/dL), and gamma glutamyl transferase (GGT) 97 (reference 12–58). Initial erythrocyte sedimentation rate was within normal limits at 18 mm/hr (reference <20 mm/h). Results of blood cultures, monospot, and hepatitis B, C, and HIV screens were negative. A CT scan showed diffuse enhancement of the gallbladder with gallbladder wall thickening (Figure, right panel). A small amount of pericholecystic fluid was seen, but no distension of the gallbladder or gallstones were noted. These findings prompted a general surgery evaluation for acute cholecystitis, but their examination results were not consistent with this diagnosis. Given the patient's flulike symptoms and laboratory abnormalities, the diagnosis of Q fever was considered. The patient had initially been treated with doxycycline and metronidazole, but metronidazole was discontinued when his physical examination results remained benign. His fever curve decreased within 2 days of receiving doxycycline. He was transferred out of theater to Landstuhl Regional Medical Center in Germany for further evaluation. Q fever was confirmed with *C. burnettii* serum titers of 2,048 for phases 1 and 2 IgM. He improved with doxycycline, 100 mg twice a day for 14 days, and was subsequently returned to duty. No exposure factors were identified.

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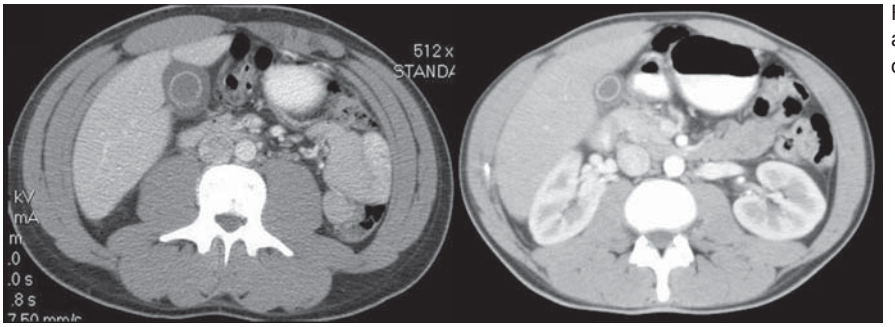

Figure. Computed tomographic scans of abdomens of 2 patients with inflammation of the gallbladder.

The third case occurred in July 2006 in a 34-year-old female active duty soldier with a history of asthma. She was seen at the troop medical clinic in Baghdad, Iraq, with flulike symptoms. She was given symptomatic treatment and released but returned with altered mental status, shortness of breath, and abdominal pain. A CT scan of her chest showed a left lower lobe infiltrate and bilateral pleural effusions. An ultrasound examination of the right upper quadrant showed no abnormalities. She was transferred to the 10th CSH in Baghdad for further care. She remained febrile (39.8°C) and tachycardic and required 4 L/min of oxygen via nasal cannula to maintain an oxygen saturation of 96%. Results of laboratory tests conducted at the time of admission were unremarkable except for a mild transaminitis (AST 139 and ALT 96). She was treated with levofloxacin, 500 mg per day intravenously, for suspected pneumonia. She had rapid worsening of her respiratory status over the next 8 hours and required intubation. Antimicrobial drug coverage was broadened to include piperacillin/tazobactam 3.375 parenterally every 6 hours; solumedrol was added, given her history of asthma. She was evacuated to Landstuhl Regional Medical Center in Germany. A bronchoscopy was performed, but results were unremarkable. Her chest radiographs showed progression to acute respiratory distress syndrome (ARDS), and arterial blood gas testing showed partial pressure of arterial oxygen to be 50–60 mm Hg. Blood, sputum, and urine cultures were negative. Doxycycline was prescribed for possible Q fever. She improved and was evacuated to WRAMC, where she was afebrile (37.2°C) at admission. Her pulmonary status improved quickly, and she was extubated. She was discharged and completed 14-day courses of levofloxacin and doxycycline. Her serologic test results were positive for Q fever with phase 2 IgM titer of 1,024. No exposure risks were identified.

Conclusions

Fever, pneumonia, and/or hepatitis are the most common signs of acute infection with Q fever (*4*,*5*). In those in whom chronic disease develops, infective endocarditis is the initial condition in >70% of cases. Asymptomatic infection may occur in >50% of infected patients (*4*,*5*). Despite its typical signs and symptoms, Q fever is known to have a multitude of clinical manifestations. Raoult described >7 distinct presentations (*6*): fever, pneumonia, hepatitis, meningitis, meningoencephalitis, pericarditis, and myocarditis. Parker et al. described >30 clinical syndromes (*4*). This broad variation can result in delayed diagnosis.

Only 12 cases of acute cholecystitis associated with Q fever have been reported in the English medical literature (*7*–*10*). The largest and most detailed description is from a case series by Rolain (*7*), who described 9 patients whose initial sign of Q fever was acute cholecystitis. Clinical data are available for only 1 other case (*8*). The most appropriate treatment for these patients remains a question. For these 10 patients, 6 had cholecystectomy. The remaining 4 and our 2 patients did well with medical management alone. Four of the 6 patients received doxycycline, 1 received ofloxacin, and 1 received no treatment. Q fever is often self-limiting; yet treatment is recommended to shorten duration of symptoms and prevent chronic disease (*5*).

Reina-Serrano recently suggested that patients with Q fever–associated cholecystitis could be managed medically (*8*). Two of our patients had evidence of cholecystitis on imaging studies but did not have evidence of peritonitis on physical examination. Our 2 patients with radiographic cholecystitis responded quickly to doxycycline. We propose that for patients with acute acalculous cholecystitis and a high suspicion for Q fever, doxycycline be given empirically. The patients' clinical response should be evident within 48 hours and surgery may be avoided. If a patient has gallstones or acute abdominal pain, a standard approach for treating acute cholecystitis should be followed.

The third patient in our series progressed to ARDS, which has been reported, albeit rarely, with Q fever (*1*,*4*,*11*,*12*). More typically, pneumonia secondary to acute Q fever infection results in a dry to productive cough, pleuritic chest pain, and focal or bilateral infiltrates on chest radiographs (*6*).

Our patients denied having typical risk factors, including exposure to livestock or consumption of local meat or dairy products. However, direct exposure to such products is not necessary (*1*,*4*,*5*). We agree with Anderson et al., who suggested that providers strongly consider adding

doxycycline to the treatment regimen for deployed soldiers with severe pneumonia (*1*).

Q fever is a Category B biologic agent and must be considered as a potential threat to deployed soldiers (*13*). The most likely mode of attack would be aerosolization; given the low dose required for infection (1–10 organisms), multiple cases would follow. We considered bioterrorism unlikely, given the limited number of clinically symptomatic cases and the lack of a cluster of cases.

Q fever continues to be a threat to deployed US soldiers in Southwest Asia. Lack of knowledge about it can delay diagnosis and treatment. It should be considered in the differential diagnosis of any deployed or recently deployed soldier with a febrile illness, especially when hepatitis or pneumonia is present.

Dr Hartzell is an infectious disease fellow at WRAMC in Washington, DC, and an assistant professor of medicine at the Uniformed Services University in Bethesda, Maryland. His research is focused on leishmaniasis and HIV.

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Streptococcus sinensis Endocarditis outside Hong Kong

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Streptococcus sinensis has been described as a causative organism for infective endocarditis in 3 Chinese patients from Hong Kong. We describe a closely related strain in an Italian patient with chronic rheumatic heart disease. The case illustrates that *S. sinensis* is a worldwide emerging pathogen.

Among the hundreds of bacteria that are pathogenic for humans, some are reposted only once and remain a rarity, while others are considered as emerging pathogens after several cases have been published. In 2002, Woo et al. from Hong Kong Special Administrative Region, People's Republic of China, reported a new pathogen isolated from a 42-year-old Chinese woman with mitral regurgitation due to chronic rheumatic heart disease and infective endocarditis (HKU4) (*1*). Its 16S rRNA sequence (*2*) showed a new streptococcal species, subsequently named *Streptococcus sinensis* in honor of China; the sequencing showed that it was closely related to *Streptococcus gordonii* (96.4% homology) and to *Streptococcus intermedius* (96.3%) (*1*). Phenotypically, the species most closely resembled *S. intermedius*; some evidence suggests that *S. sinensis* could be the common ancestor of *S. anginosus* and *S. mitis* (*3*). In 2004, the same group published a retrospective analysis of 302 bacteremia cases caused by *S. viridans* in Hong Kong, including 2 other cases of endocarditis caused by *S. sinensis* with Lancefield group F (*4*). We describe another case of an infective endocarditis (or infection in general) due to *S. sinensis* outside Hong Kong.

The Case

In December 1998, a 57-year-old Italian man with a severe mitral insufficiency of rheumatic origin underwent a nonbleeding dental procedure without antimicrobial prophylaxis. One month later, he was hospitalized at the University Hospitals of Geneva with fever (38°C–39°C), rigors, and weight loss of 4 kg in 3 weeks. There was no history of prior endocarditis, drug abuse, traumatism, or concomitant disease. At admission he was hemodynamically compensated and without fever. Over the cardiac apex, a grade 5/6 proto-mesosystolic murmur was audible. Transesophageal echocardiography showed vegetation on the mitral valve without abscesses. An ophthalmologic examination showed an embolus near the right macula. The urinary sediment exhibited microhematuria. In all blood-culture bottles, a gram-positive *Streptococcus* sp. was grown. Thus, infective endocarditis was diagnosed by the presence of 2 major and 3 minor criteria according to modified Duke criteria (*5*).

An antimicrobial drug treatment with intravenous (i.v.) penicillin G 6 × 4 million U/day and gentamicin 3 × 1 mg/kg/day for 3 weeks was initiated. After an excellent clinical course and normalization of inflammation markers, treatment was switched to i.v. ceftriaxone 1 × 2 g/day for another 3 weeks to enable outpatient therapy (*6*). No secondary abscesses occurred. Several infected teeth were extracted during treatment. Because of the severity of the preexisting mitral regurgitation, an elective replacement with a mechanical prosthesis was performed in March 1999. The patient was considered cured. Three years later he died of cerebral hematoma attributed to oral anticoagulation. Autopsy did not show any sequelae of former infection.

All 6 blood-culture bottles (3 BACTEC aerobic plus/F and 3 BACTEC anaerobic lytic/F; Becton Dickinson, Sparks, MD, USA.) were positive with gram-positive cocci in chains. The isolate grew as transparent α-hemolytic colonies (0.5- to 1-mm diameter) on sheep blood Columbia agar after an incubation of 24 h at 35°C in 4% CO_2-enriched atmosphere. The biochemical identification system API 20 Strep (bioMérieux, Lyon, France) was used to attempt the identification. The numerical profile was 4241450, indicating *S. sanguinis* (95% similarity). Agglutination with Lancefield antisera (Streptokit, bioMérieux) was negative for groups A, B, C, D, F, and G. Antimicrobial MICs were determined with Etest (AB Biodisk, Solna, Sweden). Results were interpreted according the available Clinical Laboratory Standard Institute (CLSI, formerly NCCLS) criteria (*7*) (Table).

The genetic sequence of the 16S rRNA was determined by a capillary sequence analyzer (ABI 3130 XL DNA Analyzer, Applied Biosystems (Foster City, CA, USA) and compared with the nucleotide sequences in GenBank. Of >1,000 bp in the 16S rRNA sequence, only 2 differed from the previously published *S. sinensis* HKU4 (AF432856) (*1*), a sequence identical to that of HKU5 (AF432855) and HKU6 (AF432857) (*4*). Results were positive for identifying the superoxid dismutase (*sodA)* (primer from reference *8*) and RNA polymerase β-subunit (*rpoB)* (*9*) housekeeping gene sequences. Concerning *sodA*, for >404 bp there was 97.3% homology with the positive *S. sinensis* strain

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Table. Susceptibility of *Streptococcus sinensis*–related strain from University Hospitals of Geneva

Antimicrobial agent	MIC (mg/L)	Interpretation
Penicillin	0.064	Susceptible
Ceftriaxone	0.19	Susceptible
Imipenem	0.064	Susceptible
Gentamicin	3	Intermediate resistant
Levofloxacin	1	Susceptible
Clindamycin	0.094	Susceptible
Erythromycin	0.047	Susceptible
Rifampicin	0.032	Susceptible
Cotrimoxazole	1	Susceptible
Tetracycline	0.125	Susceptible
Tigecycline	0.064	Susceptible
Linezolid	0.5	Susceptible
Vancomycin	1	Susceptible
Teicoplanin	0.25	Susceptible

AY386220 (*9*) and only 2 bp differences in >286 bp (99.3% homology) with the *S. sinensis* strain EF451825. Concerning *rpoB*, 485 bp were identical over 516 nt sequences (94% homology) of the *S. sinensis* strain AF199923 (*9*).

Conclusions

We report a case outside Hong Kong of infective endocarditis caused by a strain of *S. sinensis*. Information on this novel pathogen has been published for 3 Chinese patients (*2*–*4*). Ours is the fourth published case worldwide. Because the original *S. sinensis* (*1*) is most closely related to our strain, we believe that our isolate belongs to that species. The close relationship of the sequence of our strain to the referential *sodA* and *rpoB* gene sequences also identifies our Geneva strain as *S. sinensis*. We have submitted the *sodA* and *rpoB* sequencing results to GenBank (accession nos. EF585234 and EF591041, respectively).

A 16S rRNA sequence with only 3 base differences from HKU4 had been detected by a German group in the aortic valve of another patient (GenBank accession no. AY049738, unpub. data), and other sequences have been reported by a French group (EF371928, unpub. data) and a British group (AY386220, unpub. data). We do not know more about these cases. Our Geneva strain differed only in 2 nt bases from the HKU4 strain (on positions 43 and 48) and only in 1 base (position 66) from the German sequence, whereas it is identical with the French sequence. This might suggest the emergence of a European strain of *S. sinensis*. From a clinical point of view, all reported patients (*1*,*4*) had an underlying chronic rheumatic heart disease of the mitral valve as the major risk factor. Only l patient had a preceding tooth extraction (*4*), like our patient with teeth abscesses. No other infections besides endocarditis caused by *S. sinensis* have been described thus far in humans or in animals. All previously reported patients were successfully treated by i.v. penicillin G or ampicillin for 4 weeks (combined therapy with gentamicin during the first 2 weeks) without necessity for surgical intervention. In light of other streptococcal endocarditic recommendations (*10*,*11*) and reported experiences with *S. sinensis* (*4*), our 6 weeks of antimicrobial drug treatment was probably excessive. The elective mitral valve replacement was indicated because of the severity of the preexisting regurgitation.

In conclusion, our case outside Hong Kong confirms that *S. sinensis* causes endocarditis throughout the world. Like other viridans streptococci, *S. sinensis* might be part of the human oral flora. The real number of cases is probably underestimated because commercial kits misidentify *S. sinensis* as *S. intermedius* or *S. anginosus* (*1*). With adequate sequencing technology, further reports may indicate the real prevalence of this emerging pathogen.

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PCR versus Hybridization for Detecting Virulence Genes of Enterohemorrhagic *Escherichia coli*

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We compared PCR amplification of 9 enterohemorrhagic *Escherichia coli* virulence factors among 40 isolates (21 O/H antigenicity classes) with DNA hybridization. Both methods showed 100% of the chromosomal and phage genes: *eae*, *stx,* and *stx2*. PCR did not detect 4%–20% of hybridizable plasmid genes: *hly*A, *katP*, *espP*, *toxB*, open reading frame (ORF) 1, and ORF2.

Enterohemorrhagic *Escherichia coli* (EHEC) pathogenicity is usually linked to a Shiga toxin (*1*,*2*) and virulence factors, including adhesins, toxins, invasins, protein secretion systems, iron uptake systems, and several unidentified functions (*3*,*4*), which are unrelated to strain phylogeny. In many laboratories, sorbitol-MacConkey medium is commonly used to screen for the slow sorbitol fermentation phenotype of the most common Shiga toxin–containing strain: O157:H7 (*5*), but this process does not address the pathogenic potential of the remaining sorbitol-positive *E. coli*. These organisms can be detected by immunologic methods or PCR evaluation of virulence factors. PCR is the most useful method for virulence factor detection, and others have made convincing arguments for its use in characterizing the virulence factor patterns of potential pathogens (*6*,*7*).

Variation in virulence factor targets and use of different PCR primers contribute to variable results in detecting the most common virulence factors: *stx*1, *stx*2, *eae*, and *hlyA* (or *ehx*A). Variation in amplification success is likely to increase because more virulence factor variants are certain to emerge as more EHEC and Shiga toxin–producing *E. coli* (STEC) strains are identified. This study addresses the potential for a broad and well-characterized set of control strains relative to virulence factor amplification and confirmed by Southern hybridization.

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The Study

We used PCR amplification and Southern blot hybridization to detect 9 virulence factors among 40 EHEC type-strains from the STEC Center, National Food Safety and Toxicology Center, Michigan State University (East Lansing, MI, USA). The virulence factor targets were the following: 1 chromosomal (*eae* [*8*]), 2 phage (*stx*1 and 2), and 6 plasmid (open reading frame [ORF] 1, ORF2 of pOSAK1 [*1*,*2*]; espP [*9*], hlyA [*4*,*10*], katP [*11*], and tox B [*12*] of pO157) (Table). DNA-DNA hybridization probes were made from virulence factors amplified from O157:H7 EDL933 genomic DNA.

PCR amplification was carried out with PCR primers (20 pmol/L each per 50 μL reaction) (Integrated DNA Technologies, Coralville, IA, USA) (Table) and 1 μL genomic DNA (extracted from overnight Luria-Bertani broth cultures according to PureGene DNA isolation kit instructions [Gentra Systems, Minneapolis, MN, USA] and dissolved in 50 μL 10 mmol/L Tris, pH 8.3) in a PCR cocktail containing 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 1 U Vent exo(–) polymerase from New England BioLabs (Beverly, MA, USA), and 200 μmol/L each dATP, dGTP, dTTP, and dCTP. The mix was incubated for 30 cycles of 94°C, 40 s; annealing (for temperatures, see Table), 45 s; 72°C, 60 s, and a final 10-min extension at 72°C. Amplification products were confirmed by DNA sequencing.

^{32}P-labeled DNA probes were made from 2 μg PCR amplicons (purified by Montage PCR Cleanup Spin Column (Milipore Corp., Burlington, MA, USA). The DNA was denatured at 94°C, 40 sec; annealed (temperatures in Table) with 50 pmol/L of the appropriate PCR primers, 45 s extended for 2 h at 72°C. The 1× buffer contained the following: 1.5 mmol/L $MgCl_2$, 0.4 mmol/L each dATP, dGTP, dTTP; 2.0 μL 3,000 Ci/mmol α-^{32}P-dCTP (MP Bioscience, Buxton, UK); and 1.25 U Taq polymerase in a 50 μL final volume. Unincorporated ^{32}P-nucleotide was removed by Sephadex G-50 in Tris-EDTA, 1% sodium dodecyl sulfate (SDS).

Bacteria (800 μL overnight cultures) were transferred to Hybond-N+ nitrocellulose membrane (Amersham Biosciences UK Ltd, Buckinghamshire, UK) by dot-blot vacuum filtration apparatus (Schleicher and Schuell, Keene, NH, USA). Lysis and binding of genomic DNA fixation were carried out by exposure to lysis solution (1.5 mol/L NaCl, 0.5 mol/L NaOH) twice for 5 min each, and twice with neutralization solution (1 mol/L Tris-Cl, pH 7.4; 1.5 mol/L NaCl) for 5 min each. The filter was then submerged in 2× SSC with gentle agitation, air dried, and the DNA UV (254 nm) cross-linked at 120,000J/cm^2 (CL-1000 cross-linker, Fisher Biotech, Pittsburgh, PA, USA).

Probe hybridization was carried out in rotating hybridization bottles (Fisher Scientific Isotemp hybridization oven, Fisher Biotech) in 20 mL 6× SSC, 1% SDS at

Table. Virulence factor targets and primers, including nucleotide sequences, reference, and PCR conditions*

Primer name	Nucleotide sequence (5′→3′)	Target (bp)	Ref.	PCR conditions		
				Denature	Anneal	Extension
STX1U	GTAACATCGCTCTTGCCACA	Stx1 gene (204)	This study	95°C, 60 s	53.7°C, 60 s	72°C, 240 s
STX1D	CGCTTTGCTGATTTTTCACA					
STX2U	GTTCCGGAATGCAAATCAGT	Stx2 gene (206)	This study	95°C, 60 s	53.7°C, 60 s	72°C, 240 s
STX2D	CGGCGTCATCGTATACACAG					
eae-1	ACGTTGCAGCATGGGTAACTC	Intimin (818)	(*8*)	95°C, 60 s	57.1°C, 60 s	72°C, 240 s
eae-2	GATCGGCAACAGTTTCACCTG					
ToxBF	TGGCCTTGCGCTCTATAAGAACCT	ToxB (823)	This study	95°C, 60 s	60°C, 60 s	72°C, 240 s
ToxBR	ACCACGCCGTGAGAATAATGTCCA					
HlyA1F	GGTGCAGCAGAAAAAGTTGTAG	HlyA (1551)	(*13*)	95°C, 60 s	55.5°C,60 s	72°C, 240 s
HlyA1R	TCTCGCCTGATAGTGTTTGGTA					
EspPF	CGGCAGAGTATCATCAAGAGC	EspP (397)	This study	95°C, 60 s	55.5°C, 60 s	72°C, 240 s
EspPR	CATTAAATGGAGTTATGCGTC					
KatPF	TTTAAAACGCTGGGATTTGC	KatP (1174)	This study	95°C,60 s	52.0°C, 60 s	72°C, 240 s
KatPR	CTCCTGAGAGGCGTCAGTTC					
MalBU	GACCTCGGTTTAGTTCACAGA	MalB promoter (414)	This study	95°C, 60 s	55.8°C, 60 s	72°C, 240 s
MalBDn	AGCGCGTAGGACTGAAACACCATA					
ORF1F	TTTTTCAAAGCAAATGATGTGG	ORF 1 pOSAK1 (385)	This study	95°C, 60 s	49.8°C, 60 s	72°C, 240 s
ORF1R	GGCGTAGCTAGGTTGAAATTATG					
ORF2F	CAA CCTAGCTACGCCACCAT	ORF 2 pOSAK1 (869)	This study	95°C, 60 s	54.3°C, 60 s	72°C, 240 s
ORF2R	CATCAGGCGGAAATACCACT					
EAF1	CAGGGTAAAAGAAAGATGATAA	Eaf (397)	(*14*)	95°C, 60 s	49.8°C, 60 s	72°C, 240 s
EAF2	TATGGGGACCATGTATTATCA					
BFP1	GATTGAATCTGCAATGGC	Bfp (597)	(*15*)	95°C, 60 s	51.6°C, 60 s	72°C, 240 s
BFP2	GGATTACTGTCCTCACATAT					

*Ref., reference; ORF, open reading frame.

68°C. Membranes were washed twice, for 1 min, in room temperature 2× SSC, 0.1% SDS, and twice at 45°C for 1 h in 1× SSC, 1% SDS. Hybridized membranes were exposed overnight with a phospho-imaging screen (Bio-Rad, Hercules, CA, USA) and visualized with a Personal Molecular Imager FX (Bio-Rad). The 3 chromosomal targets (*stx*1, *stx*2, and *eae*) were detected with 100% efficiency by both PCR and hybridization (no. positive by PCR/no. positive by hybridization): 21/21 *stx*I, 19/19 *stx*II, and 37/37 *eae*. Plasmid-associated genes, however, were detected with less efficiency relative to hybridization: *katP*: 15/17 (88%), *hly*A: 26/27 (96%), *espP*: 19/23 (83%), *toxB*: 13/16 (81%), and both ORF1 and ORF2: 4/5 (80%) (online Appendix Table, available from http://www.cdc.gov/EID/content/13/8/1253-appT.htm).

Seventy-five percent (30/40) of the pathogenic *E. coli* strains tested contained at least 1 *stx* gene, 23% (9/40) were positive for both *stx*1 and *stx*2. The most common gene detected was intimin (*eae*), which was positive by both PCR and hybridization in 37/40 (93%) of the strains. While *eae* is strongly correlated with Shiga toxin, the adherence phenotype conveyed may be sufficient to cause a pathogenic state because 4 of the clinical isolates investigated contained only the *eae* gene.

Six plasmid virulence factor genes of pO157 and pOSAK1were targeted. Thirty-one (78%) of the 40 pathogenic strains tested were positive for at least 1 (by hybridization, PCR, or both) of the 4 genes, *toxB*, *katP*, *hlyA*, *espP*, which are usually carried on the archetypal pO157 plasmid: 11/31 (35%) retained all 4, 4/31(13%) carried three, 9/31 (29%) carried 2, and 5/31 (16%) carried only 1 (the sole PCR-positive/hybridization-negative isolate [*esp*P in ED-31] was presumed to result from a nonspecific amplification).

Five EHEC strains (13%) hybridized to both ORF1 and ORF2 (from pOSAK1) (*1,4)*, but only 4 were amplifiable. These same 4 also contained *eae* and at least 1 Shiga toxin gene (the 1 that failed to amplify [E851/71] lacked both *stx*1 and *stx*2).

Conclusions

The current accepted standard for EHEC identification is amplification of *stx1*, *stx2*, *eae*, and *hlyA* by PCR. However, this technology is generally only available at large hospital or state health laboratories. Hybridization is superior to culture screening methods and largely complimentary to PCR, but has a potentially broader epidemiologic application since it is unaffected by minor sequence variations that can completely inhibit PCR.

Only 3% of the 360 virulence factor hybridizations made in this study did not amplify. PCR failure is expected with its relatively higher sensitivity to single base primer-hybrid mismatch compared to whole amplicon hybridization. Notably, however, all 12 variations detected were among plasmid-associated virulence factors: 95%

(228/240) of the plasmid hybridizable targets were amplified, compared to 100% (120/120) of the hybridizable chromosomal targets.

Although we detected the variable presence of genes ostensibly associated with 2 plasmids (pO157 and pOSAK1) and the bacterial chromosome, we did not attempt to verify either plasmid or chromosomal locations for any of the amplicons or DNA:DNA hybrids. While all virulence factor targets summarized in this study are subject to change there have been reports of any of the putative chromosomal or plasmid virulence factor targets in this study being found elsewhere.

Prager et al. (*7*) recently reported, using PCR alone, a wide variety of 25 virulence factor combinations among 266 pathogenic *E. coli* isolates representing 81 serotypes. Such diversity speaks directly to the need to accurately assess virulence factor presence to evaluate epidemiologic and clinical correlations. A similar 5% failure of the plasmid-associated virulence factor amplifications could have implications in such virulence factor correlations. Overall, however, these results are very similar to those of this study of prospective control strains. The use of a single control, such as EDL 933, will inherently bias PCR detection schemes since a failure of amplification in a test will be read as the absence of virulence factor element because it was amplifiable in the control.

If amplification failure is a measure of template variation, we find a much greater variability among plasmid-associated virulence factors. Although pO157 has been reported in most O157 H7 strains (*13*), our study demonstrates a high variability in the putative virulence factor content of pO157 as well as a highly variable content of pO157-associated virulence factors among the O157 isolates screened. Finally, pO157-associated virulence factors were detected among all but 4 of the 20 *E. coli* serotypes examined.

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Mr Gerrish completed this work as part of his master's thesis at Idaho State University. He is currently a doctoral candidate in microbial pathogenesis at the State University of New York at Buffalo.

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Multidrug-Resistant Bacteria in Southeastern Austria

To the Editor: In many parts of the world, the proportions of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and extended-spectrum β-lactamase (ESBL)–producing organisms in the family *Enterobacteriaceae* have increased remarkably during recent years (*1*). However, proportions of antimicrobial drug resistance vary substantially at national and regional levels. We describe antimicrobial drug resistance data for hospitalized patients and outpatients in southeast Austria.

A total of 690,967 clinical samples were collected from hospitalized patients and outpatients and analyzed at the microbiology laboratory of the Medical University of Graz during 1997–2006. Selected for resistance surveillance were nonduplicate isolates of *S. aureus, Enterococcus faecium, E. faecalis, Escherichia coli,* and *Klebsiella* spp. Antibiotic susceptibilities were determined by using disk diffusion and the VITEK2 system (bioMérieux, Marcy l'Etoile, France) with specific susceptibility test cards. Etest (AB Biodisk, Solna, Sweden) was used to confirm results. Test results were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (*2*).

During the study period, the proportion of patients with MRSA remained stable (2.5%–4.9%) (Figure, panel A). The prevalence of MRSA among invasive *S. aureus* isolates ranges between 0.5% and 44.4% in European countries and has increased in recent years (*3*). We found MRSA predominantly in samples from hospitalized patients (median 72.1%); however, the incidence of community-acquired MRSA increased slightly during recent years, similar to that of other central European countries (*4*). Vancomycin resistance was not noted during the study period; however, 4 vancomycin-intermediate MRSA isolates were noted in 2004, 2005, and 2006, in concordance with the sporadic occurrence of MRSA with intermediate susceptibility to glycopeptides recently reported for other European countries (*3*).

The percentage of patients with vancomycin-resistant *E. faecium* and *E. faecalis* was low (median 0.4%) (Figure, panel A). In total, 10 *E. faecium* and 4 *E. faecalis* isolates with resistance to vancomycin were reported; most were from hospitalized patients. As in most European countries, human infections due to glycopeptide-resistant enterococci remain rare in Austria, although a high proportion of glycopeptide-resistant *E. faecium* was reported recently from animals used in food production (*5*).

Among *E. coli* isolates, no ESBL producers were noted in 1997. From 1998 through 2002, proportions of ESBL-producing *E. coli* were 0.06%–0.13%, which corresponds to 3–6 isolates per year. A subsequent increase of ESBL-producing *E. coli* isolates was noted, from 19 (0.3%) in 2003 to 148 (2.4%) in 2006 (Figure, panel B). Most (67%) ESBL-producing isolates found during 2003–2006 originated from community-acquired urinary tract infections. Resistance of *E. coli* to carbapenems was not reported during the study period. Among *Klebsiella* spp. isolates, 2 (0.2%) ESBL producers were observed during 1997. From 1998 through 2004, the prevalence of

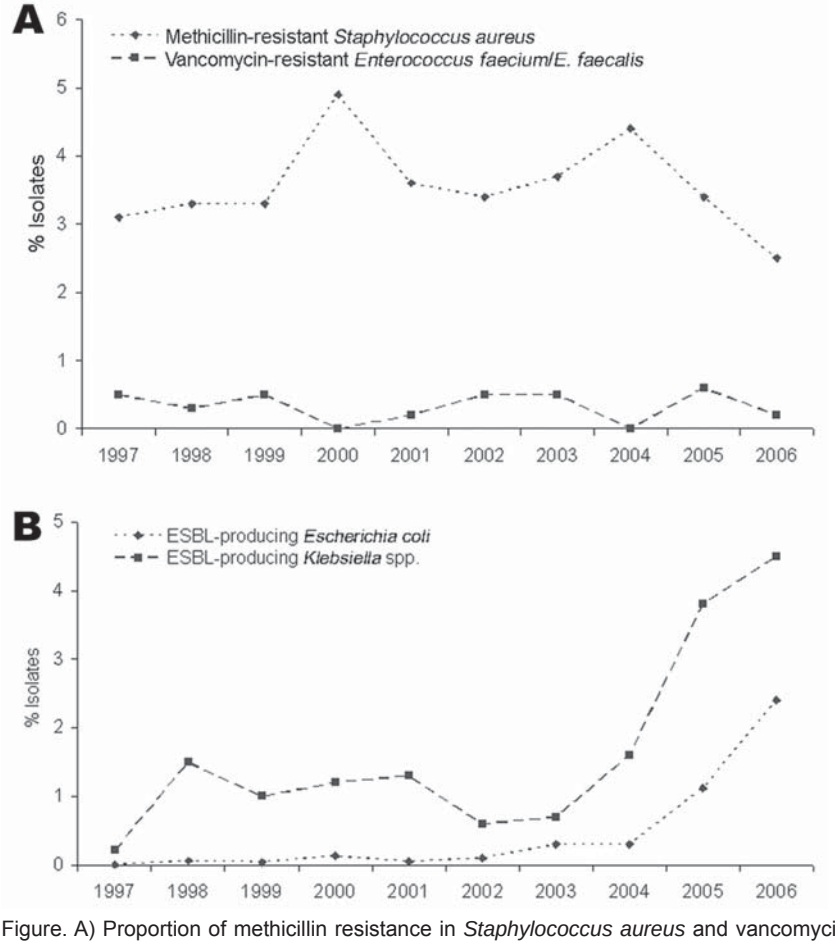

Figure. A) Proportion of methicillin resistance in *Staphylococcus aureus* and vancomycin resistance in *Enterococcus faecium* and *E. faecalis* in southeastern Austria, 1997–2006. B) Proportion of extended-spectrum β-lactamase–producing (ESBL) *Escherichia coli* and *Klebsiella* spp. in southeastern Austria, 1997–2006.

ESBLs among *Klebsiella* spp. ranged between 0.6% and 1.6%. In 2005 and 2006, the rate of ESBL-producing *Klebsiella* spp. increased to 3.8% (44 isolates) and 4.5% (55 isolates), respectively, and originated mainly from intensive care units (Figure, panel B). In 2005, a single *Klebsiella pneumoniae* isolate showed reduced susceptibility to imipenem (MIC 2 μg/mL) and to meropenem (MIC 4 μg/mL) and resistance to ertapenem (MIC >16 μg/mL). Nevertheless, production of ESBL by *Enterobacteriaceae* organisms is still rare in southeast Austria compared with other European countries (*6*). However, a dramatic increase of ESBL-producing *E. coli* and *Klebsiella* spp. has been observed during recent years.

The increase of ESBL-producing *E. coli* isolates in outpatients with urinary tract infections leads to serious treatment problems. Results from a recent study indicate that the increase of ESBL-producers in southeast Austria is caused mainly by the emergence of CTX-M–type ESBLs, which are increasingly being isolated from outpatients (*7*). The *K. pneumoniae* isolate found in 2005 represents the first ESBL-producing isolate not susceptible to carbapenems reported from Austria. Development of resistance to carbapenems in *Enterobacteriaceae* organisms has been reported increasingly, which substantially limits treatment options for persons with multidrug-resistant gram-negative infections (*8*).

Our data show insignificant changes in prevalence of MRSA and vancomycin-resistant enterococci in southeast Austria during the past decade but an alarming increase of multidrug-resistant ESBL-producing *E. coli* and *Klebsiella* spp. isolates in recent years. Detection of an ESBL-producing *K. pneumoniae* isolate with reduced susceptibility to carbapenems shows that pathogens with new mechanisms of resistance are emerging in this region.

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Osteomyelitis of Parietal Bone in Melioidosis

To the Editor: In Europe and the United States, melioidosis is a rare disease, with no cases reported thus far from Slovenia. However, it is a relatively common disease in certain areas of Southeast Asia and northern Australia. Potentially fatal, this disease is caused by the gram-negative bacillus *Burkholderia pseudomallei*, an environmental organism found in the soil and water of disease-endemic areas. Human infections are mostly acquired through percutaneous inoculation during contact with contaminated water and soil, although inhalation is also a recognized route of acquisition (*1*). Heavy monsoon rain is associated with severe disease course (*2*). Melioidosis was reported in some persons injured in the Tsunami in 2004 (*3*). The disease has a wide spectrum of signs and symptoms (*4*). Osteomyelitis is a rare manifestation. It occurs in <5% of cases and is a clinical challenge to diagnose and treat (*1*,*4*,*5*).

We describe a case of melioidosis in a previously healthy, 40-year-old Slovenian man. The patient had been working as a basketball trainer in Jordan for the previous 12 months and was traveling to Brunei in mid-summer 2006, 14 days before the illness started. While visiting Brunei, he sustained a minor head trauma when he hit his head on a night table at the hotel. Ten days later, high-grade fever up to 40°C developed, without any other signs or symptoms of disease. After returning to Jordan, the patient was admitted to a local hospital and received different antimicrobial agents without any improvement of his medical condition. After 6 weeks of unsuccessful treatment, he decided to continue medical treatment in Slovenia.

On admission to our hospital, he reported headache and persistent high fever of 6 weeks' duration. Physi-

cal examination indicated high fever (39.5°C) and occipital swelling without any neurologic deficits or other abnormal findings. Initial complete blood cell count, liver function test results, blood urea nitrogen levels, and creatinin levels were normal. C-reactive protein was 60 mg/L, and erythrocyte sedimentation rate was 47 mm/h. Results of chest radiograph and abdominal ultrasound were normal. Results of repeated blood cultures and urinalysis were negative.

The suspected clinical diagnosis was brucellosis (the patient had eaten unpasteurized soft cheese during his stay in Jordan, and brucellosis is endemic in the Middle East). While waiting for *Brucella* spp. tests, we began empirical antimicrobial drug treatment with doxycycline. The patient's condition improved promptly. He became afebrile after 4 days of therapy. In the following week, ultrasound of occipital area soft tissue was performed, and posttraumatic seroma was diagnosed. *B. pseudomallei* was isolated from the seroma on sheep blood agar and identified with VITEK 2 gram-negative identification card (bioMérieux, Marcy l'Etoile, France). The isolate was sensitive to piperacillin, piperacillin-tazobactam, ceftazidime, imipenem, meropenem, and chloramphenicol. It was resistant to aminoglicosides (gentamicin, tobramycin, amikacin, netilmicin), colistin, and polymyxin B. Etest MIC showed susceptibility to doxycycline (MIC 2 μg/mL) and trimethoprim/sulfamethoxazole (TMP/SMX) (MIC 1/19 μg/mL). Susceptibility of *B. pseudomallei* to TMP/SMX was tested with Etest because the disc-diffusion method is inappropriate and can overestimate the extent of resistance (*6*).

The patient later recalled going on a jungle trip in Brunei the day after his accident. During the trip, he scratched his head, and the skin started to bleed. Thus, he likely inoculated bacteria into the subcutaneous tissue of the head. Fever developed 10 days later.

Computed tomography of the scalp was performed (Figure), and osteomyelitis of the right parietal bone was detected. Magnetic resonance imaging (MRI) excluded involvement of intracranial tissues. Doxycycline was stopped and, as recommended, treatment with ceftazidime and oral TMP/SMX was started. The patient received 8 weeks of intensive parenteral therapy. Once he was discharged, he received 4 months of oral eradication therapy with TMP/SMX and doxycycline. The outcome was excellent. He is now without signs and symptoms of disease, has normal laboratory test results, and has no signs of inflammation on MRI.

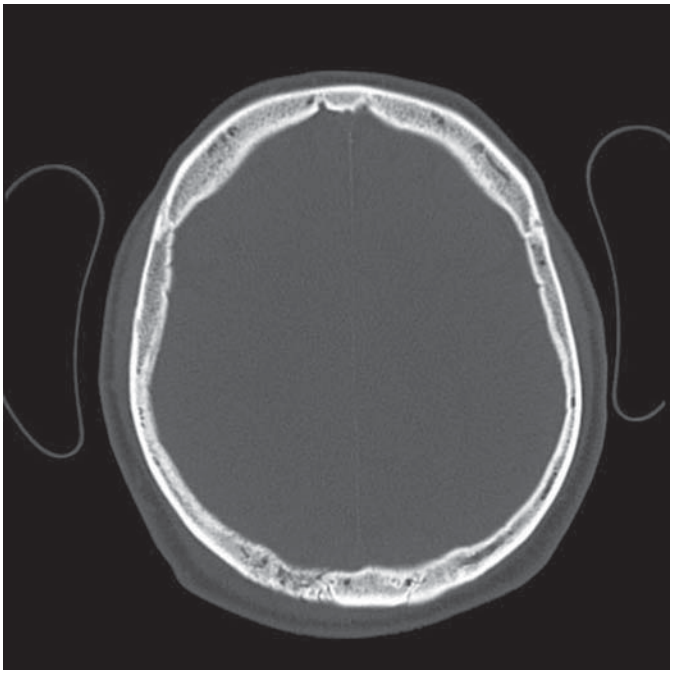

Figure. Axial nonenhanced computed tomographic scan showing moth-eaten appearance of right parietal bone characteristic of osteomyelitis.

Involvement of the skin and soft tissue is common in melioidosis (*7*). Osteomyelitis is a rare manifestation, usually part of a disseminated infection involving metaphyseal regions of long bones and vertebral bodies. Localized bone involvement is very rare (*8*). In a Thailand group of 21 patients with musculoskeletal melioidosis, all were initially treated with surgical debridement, followed by long course of antimicrobial therapy (*9*). A single report of parietal bone osteomyelitis was found in the literature; it was connected to a cerebral abscess due to hematogenous dissemination (*10*). Because of the specific location of the osteomyelitis (close to the leptomeninges), nonextensive bone damage, and good initial response to antimicrobial therapy, we decided on conservative therapy only.

Melioidosis, although a rare disease, should be considered in the differential diagnosis of any febrile illness in patients returning from disease-endemic regions, especially Thailand and northern Australia. Without special awareness of this possibility, microbiologic laboratories in nonendemic regions could likely misidentify the bacteria and consequently misdiagnose the organism.

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EID Online
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Chikungunya Fever, Andaman and Nicobar Islands, India

To the Editor: The outbreak of chikungunya fever that started in the Indian Ocean Islands in early 2005 (*1*) spread through adjoining islands and appeared in peninsular India by late 2005 (*2*). It was first noticed in the southern state of Andhra Pradesh in February 2006; it spread to Tamil Nadu in April 2006 and to Karnataka and Kerala in May. The western state of Gujarat also reported cases in April, but no cases were reported in May and June. The disease again reappeared in July and reached a peak in August. Later it affected the central Indian states of Maharashtra and Madhya Pradesh. In most states, the outbreak declined by October 2006 (*3*,*4*).

Andaman and Nicobar Islands, a union territory of India, is an archipelago of >500 islands and islets situated in the Bay of Bengal, 1,200 km from peninsular India. People are constantly moving between mainland India and these islands. Chikungunya fever has previously not been reported from these islands.

During July and August 2006, medical professionals noticed an increase in the number of cases of febrile illness in Port Blair, the headquarters of the union territory and the only urban area in the islands. The total number of patients with fever who visited the 5 urban health centers (UHC) in the town went up from the baseline of 300–450 per day to 550–900 per day in July and August 2006. Most of the patients had associated joint pain. In view of the clinical features suggestive of chikungunya fever, the ongoing epidemic on mainland India, and the widespread presence of the vector, *Aedes aegypti,* within the urban area of Port Blair (*5*), chikungunya fever was suspected. To confirm this hypothesis, 17 persons who fulfilled the case definition of having an acute febrile illness associated with severe pain in multiple joints were selected from among the initial patients who went to the UHCs and the referral hospital in Port Blair. Among these study participants, 15 were adults and 2 were adolescents 15 years of age; 6 were female and 11 male. Four adults had febrile illness associated with joint pain; in these patients, weakness of all 4 limbs developed 3–15 days after onset of illness. All of the 4 patients with weakness had areflexic quadriplegia; 1 required ventilatory support. The patients with areflexic quadriplegia were treated with injections of methylprednisolone; all recovered within a week.

Blood samples were collected from these study participants. Serum samples were separated and sent to the National Institute of Virology, Pune, for detection of anti–chikungunya virus (CHIKV) immunoglobulin M (IgM) antibodies. Samples were collected from 12 patients >4 days after the onset of symptoms. In the remaining patients, the interval between onset of symptoms and collection of blood samples was <4 days. Of the 17 study participants, 13 were positive for anti-CHIKV IgM antibodies. Three of 4 samples that were negative for IgM antibodies to CHIKV were collected <3 days after the onset of symptoms. Among these, 2 samples were subjected to reverse transcriptase–PCR by using the primers CHIKV/E1S (5′-TAC CCA TTC ATG TGG GGC-3′) and CHIKV/E1C (5′-GCC TTT GTA CAC CAC GAT T-3′), as described by Hasebe et al. (*6*); both were positive for CHIKV RNA. All these samples were tested for dengue IgM antibodies by using SD Bioline Dengue IgM Rapid Test (Standard Diagnostics Inc., Kyonggi-Do, South Korea), which uses a mixture of dengue recombinant envelop proteins and can detect all of the 4 dengue serotypes. None of the samples tested positive for dengue antibodies. Hence, CHIKV infection was confirmed in 15 of 17 patients.

India experienced the first confirmed outbreak of chikungunya fever in 1963–1964 in Kolkata (*7*) and in 1965 in Chennai. The last epidemic in India was reported from Barsi in the state of Maharastra in 1973 (*8*). However, during these outbreaks, Andaman and Nicobar Islands were not affected. Outbreaks of dengue fever and chikungunya fever are known to occur simultaneously, as has happened in several parts of India. However, during the current outbreak in Andaman Islands, dengue infection was not detected. (Dengue has never been reported in the islands.) As chikungunya fever is known for its mysterious pattern of dramatic outbreaks interspersed by periods of prolonged absence, the introduction of this virus to an unexposed population has great public health importance.

This outbreak could be a warning about preparedness for health authorities not only in these islands but also in other areas where chikungunya fever has not occurred previously. With the extent of human travel to and from areas with active chikungunya virus transmission, many areas where the disease has not previously been reported could be at risk. As an outbreak response, the Regional Medical Research Centre and Directorate of Health Services, Andaman and Nicobar Administration, has undertaken a comprehensive community-based survey to assess the impact of chikungunya fever and *Aede*s infestation levels. We are stepping up our applied field research to prevent future outbreaks of chikungunya fever, as well as dengue fever.

Acknowledgments

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Alistipes finegoldii in Blood Cultures from Colon Cancer Patients

To the Editor: *Alistipes finegoldii* was previously isolated from appendiceal tissue samples in children with acute appendicitis and from perirectal and brain abscess material (*1*,*2*). 16S rDNA sequencing studies showed that this bacterium clustered with *A. putredinis* (Figure) in the Bacteroidetes group (*4*). We describe the first cases, to our knowledge, of bacteremia due to *A. finegoldii* in 2 patients with colon cancer who underwent surgical resection.

The first patient was a 61-year-old woman with colorectal carcinoma and liver metastasis, who underwent chemotherapy consisting of 6 cycles of oxaliplatin (the FOLFOX scheme, a chemotherapy regimen consisting of fluorouracil [5 FU], folinic acid, and oxaliplatin). In September 2003, a left colectomy, resection of metastasis in the left side of the liver, and a ligation of the right portal vein were performed. Two months later, in a second step, a right hepatectomy was done. On postoperative day 5, the patient had a fever up to 39.8°C and leukocyte count of 8.49 g/L (68% polymorphonuclear leukocytes). Two blood cultures were performed before antimicrobial drug therapy based on amoxicillin/clavulanic acid and amikacin was started. After receiving this therapy, the patient recovered rapidly. One of the 2 anaerobic blood cultures was positive. Gram-negative bacilli were isolated (strain 3302398). Antimicrobial susceptibility testing showed decreased susceptibility to vancomycin, cefotetan, and penicillin G. The strain produced β-lactamase as determined by Cefinase test (Becton Dickinson, Le Pont de Claix, France).

The second patient was a 64-year-old man with colon cancer who was receiving palliative chemotherapy

(16th cycle, FOLFOX scheme); he was seen in March 2004 with a fever up to 39°C. An adenocarcinoma of the ileum had been diagnosed in June 2002 in this patient, and an ileocecal resection was performed followed by adjuvant chemotherapy. One year later, a local recurrence and peritoneal carcinomatosis were detected. The patient again underwent abdominal surgery by resection of ileo-colic anastomosis and sigmoid and peritoneal masses; a colostomy had to be created. The patient's leukocyte count was 14.94 g/L (84.6% polymorphonuclear leukocytes), and his C-reactive protein level was 268 mg/L. Before antimicrobial drug therapy with amoxicilline/clavulanic acid and ciprofloxacin was begun, blood cultures were taken. One of the 2 anaerobic blood cultures was positive. Gram-negative bacilli were isolated (strain 4401054). Antimicrobial drug resistance was detected only to vancomycin. After receiving this therapy, the patient recovered rapidly.

Biochemical characterization was conducted by using API 20A and rapid ID 32A strips (bioMérieux, Marcy l'Etoile, France). Results were compared with those obtained for the reference strain *A. finegoldii* CIP 107999^T. Strains 3302398 and 4401054 were indole positive and bile resistant, and they had positive enzyme reactions for N-acetyl-β-glucosaminidase, α-galactosidase, and β-galactosidase, as described for *A. finegoldii* (*4*). The 2 strains produced a brown pigment after 2 weeks' incubation on sheep blood agar plates (bioMérieux).

PCR amplification of the 16S rDNA was performed with the primer pair fD1/rp2 (*5*). The generated fragments were sequenced as previously described (*6*). Sequences were compared with those available in GenBank databases by using BLAST (www.ncbi.nlm.nih.gov/blast). They showed a 97% identity to the 16S rDNA of *A. finegoldii* (accession nos. AY643083 and AY643084).

A novel bacterium was characterized from appendiceal tissues samples from children with appendicitis and in 2 cases of perirectal and brain abscesses associated with other anaerobes (*1*). With routine tests, this organism resembled members of the *Bacteroides fragilis* group; however, the cellular fatty acid composition dominated by *iso*-C15:0 and production of brown pigment on media containing hemolyzed blood suggested that the organism was most closely related to the genus *Porphyromonas* (*1*). However, 16S rDNA sequence comparison showed highest sequence relatedness with *B. putredinis,* and the reclassification of *B. putredinis* in a novel genus, *Alistipes*, and the classification of the novel bacterium as *A. finegoldii* were proposed (*4*). *A. putredinis* was characterized in the indigenous flora of the human gut (*7*). The natural habitat of *A. finegoldii* is unknown but is probably the same. *B. fragilis* is the most frequent anaerobic bacterium isolated from blood samples, and the principal source of the bacteria is the gastrointestinal tract (*8*). Predisposing factors to *Bacteroides* species bacteremia include malignant neoplasms, recent gastrointestinal or obstetric-gynecologic surgery, intestinal obstruction, and use of cytotoxic agents or corticosteroids (*8*). In both of our patients, fever was noted and no other microorganisms were isolated, indications that the bacteria probably were pathogenic.

Phenotypic identification of *Alistipes* sp. is difficult in a routine microbiology laboratory. However, a molecular approach based on 16S rRNA gene sequence comparison is a good method for identifying anaerobic bacteria, as it has recently been reported for *B. fragilis* in anaerobic sepsis (*9*) and for *B. thetaiotaomicron* from a patient with a cholesteatoma and purulent meningitis (*10*). In our 2 patients, we also used molecular identification because *A. finegoldii* was not included in the API phenotypic database identification. *A. finegoldii* should be considered as an agent of bacteremia in patients with gastrointestinal pathologic conditions.

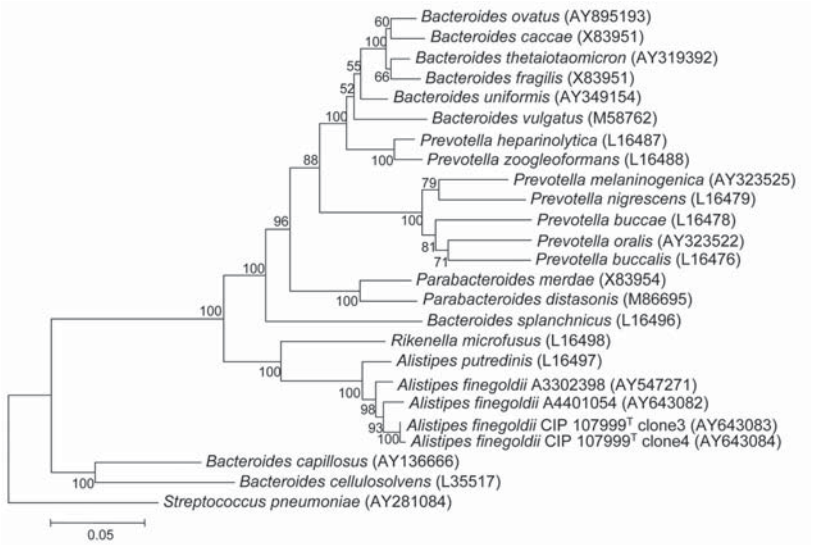

Figure. Phylogenetic tree inferred from comparison of the 16S rRNA gene sequences of genera *Bacteroides*, *Parabacteroidetes*, *Prevotella,* and *Alistipes*. Nucleotide accession numbers for the sequences used to construct this dendrogram are given in parentheses. The tree was constructed with MEGA version 2.1 (www.megasoftware.net). Distance matrices were determined following the assumptions described by Kimura (*3*) and were used to elaborate the dendrogram with the neighbor-joining method. Bar, 0.05-nt change per nucleotide position. *Streptococcus pneumoniae* was used as the outgroup.

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Shiga Toxin–producing *Escherichia coli*, Idaho

To the Editor: Data collected from expanded surveillance study suggest that more than half of Idaho Shiga toxin–producing *Escherichia coli* (STEC) illnesses are caused by non-O157 serotypes. Using data from a regional medical center whose stool culture protocol included Shiga toxin testing, we predicted Idaho's STEC incidence to be significantly higher if non-O157 STEC *E. coli* were routinely detected by immunoassay. Recent findings suggest that the prediction was accurate in an expanded surveillance area.

Several studies have shown an increased incidence of non-O157 STEC infections in the United States. For example, a community hospital in Virginia detected non-O157 serotypes in 31% of patients with STEC from 1995–2002 (*1*). A 1998 Nebraska study that analyzed 30,000 diarrheal stool samples found that non-O157 and O157:H7 STEC were equally prevalent (*2*). Additionally, findings from a Connecticut study of laboratory-confirmed cases (*3*), STEC surveillance results from Montana (*4*), and a recent study from Michigan (*5*) indicate that non-O157 serotypes comprise a substantial percentage of STEC cases.

In other countries, nonculture-based methods are routinely used for STEC detection (*6*). However, *E. coli* O157:H7 culture methods remain the focus in the United Kingdom, Canada, and the United States (*6*). Reliance on culture methods can result in misleading interpretations of STEC prevalence. For example, 93% of STEC infections in Canada are reported to be *E. coli* O157:H7, yet a Manitoba 1992 study showed that when toxin assays were used, 35% of the recovered STEC isolates were non-O157 serotypes (*6*).

Analysis of reported non-O157 STEC cases in Idaho showed a similar trend. From 2002–2004, 66% of Idaho's non-O157 cases originated in Health District 7, where >70% of stool cultures are screened by enzyme immunoassay (EIA) for Shiga toxin (Premier EHEC, Meridian Bioscience, Cincinnati, OH, USA). This rate was disproportionately higher than that of the remaining 6 health districts, which primarily use culture methods to screen for *E. coli* O157:H7. We hypothesized that this disproportion was due to differences in stool culture protocol. To test this premise, we conducted enhanced surveillance for 16 months in a "low" STEC incidence area, Health District 5. A total of 2,065 stools submitted for culture were screened for Shiga toxin by EIA. With this approach, reported non-O157 STEC incidence rose from <1 case/year/100,000 population to 11 cases/year/100,000 population. Additionally, 56% of recovered STEC isolates were non-O157 serotypes, mirroring the proportion of non-O157 detected in District 7. Notably, this appears to be the endemic rate for District 5 because no non-O157 STEC outbreaks or matching pulsed-field gel electrophoresis patterns were detected during the surveillance period. Although our study captured only a portion of stool cultures in Idaho, our findings

demonstrated increased prevalence of non-O157 STEC in the region when nonculture methods were used.

Two barriers cited for not routinely screening diarrheal stools for Shiga toxin are cost and perception of low non-O157 STEC incidence. While toxin testing is more expensive than culture testing, the potential effects of misdiagnosis may outweigh cost concerns. A study estimating the financial repercussions of *E. coli* O157 infections in the United States suggested that annual cost associated with this pathogen is $405 million, with the cost per case varying from $26 for those who do not seek medical care to $6.2 million for a patient with fatal hemolytic uremic syndrome (HUS) (*7*). Non-O157 STEC infections have been an important cause of HUS in many countries. For example, a 3-year prospective study in Germany and Austria reported that non-O157 serotypes comprised 90 (43%) of 207 STEC isolates from stools of 394 pediatric patients with HUS (*8*). Further, a 6-year Danish study of 343 registered STEC patients found that 76% of STEC and 48% of HUS cases were attributable to non-O157 serotypes (*9*). In the United States, continued reliance on O157 STEC culturing hinders our ability to determine the financial effects and the proportion of HUS cases attributable to non-O157 STEC.

Some evidence suggests that the testing focus may be changing in the United States. We used US Census Bureau population statistics to translate reported O157:H7 and non-O157 STEC cases for each state into incidence data. Despite widespread variation in STEC testing and incidence among states, there has been a significant statistical decline in the proportion of *E. coli* O157:H7 among total STEC cases every year since 2001 (Figure; $p<0.001$) (*10*). Consistent with this trend, the incidence of non-O157 STEC in the United States has increased (*10*). This may indicate that more laboratories are adopting Shiga toxin testing protocols, as we are advocating in Idaho. Our findings suggest that perceptions of low non-O157 STEC incidence in Idaho are probably artifactual and due to overemphasis on culture methods for O157 STEC. Our ongoing EIA-based surveillance highlights the need for continued investigation of the epidemiology of non-O157 STEC disease. We conclude that O157 STEC culturing has limited usefulness in areas like the Idaho health districts investigated, where non-O157 serotypes accounted for 55% of STEC illnesses. The true involvement of non-O157 in STEC disease will remain obscured as long as screening methods focus on traditional culture methods.

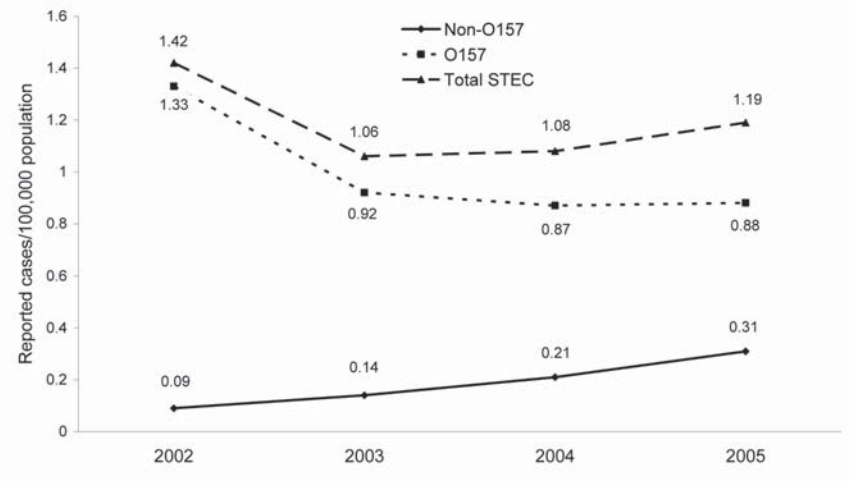

Figure. Shiga toxin–producing *Escherichia coli* (STEC) incidence trends, United States, 2002–2005.

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Imported Chikungunya Infection, Italy

To the Editor: Chikungunya virus (CHIKV) infection is a self-limiting illness characterized by fever, headache, weakness, rash, and arthralgia. Some patients have prolonged weakness or arthralgia lasting several months. In 2006, several Indian Ocean states and India had an outbreak of CHIKV infection (*1*,*2*). During the epidemic's peak, some European and American travelers returning from these areas were infected (*3*–*6*).

Because the foci of *Aedes albopictus,* 1 of the 2 main vectors of CHIKV, are now in Italy and many travelers visit CHIKV-epidemic areas, surveillance for imported cases is mandatory in Italy (*7*). From July to September 2006, a total of 17 confirmed cases of CHIKV infection were observed in travelers at 5 Gruppo di Interesse e Studio delle Patologie di Importazione (GISPI) centers (Italian network of Institutes of Infectious and Tropical Diseases). Serologic diagnosis was performed with a hemagglutination-inhibition test and confirmed by a plaque-reduction neutralization test (*8*). Demographic and epidemiologic characteristics of these patients are reported in the Table.

Cases were distributed throughout the year with a peak from March to May 2006 (n = 10). Nine patients (53%) were men. Median age was 43 years (range 31–66 years). Several reasons for travel were reported: tourism (64.6%), visits to relatives or friends (11.8%), business (11.8%), and missionary work (5.9%). One patient was a resident in the disease-epidemic area. The median exposure time in the CHIKV-endemic area for the 15 travelers was 15 days (range 9–93 days) (missionary and resident patients were excluded). The median delay before being seen at a clinic after return was 2 days (range 0–73 days). Only 7 patients (41.2%) were hospitalized. The remainder were outpatients.

All patients had fever; arthralgia (88.2%, n = 15), weakness (70.6%, n = 12), headache (11.8%, n = 2), diarrhea (11.8%, n = 2), and gum bleeding and epistaxis (5.9%, n = 1) were other reported symptoms. The median duration of fever was 5 days (range 2–12 days). Only 7 of 16 patients (43.8%) were still febrile when first seen. Physical examination showed diffuse macular erythematous rash in 13 patients (76.5%), a similar rate to that reported among French travelers (*4*). Hepatomegaly was found in 2 patients (11.8%), splenomegaly in 2 (11.8%), and peripheral lymphadenopathy in 2 (11.8%).

Twelve acute-phase patients were admitted to the hospital for blood testing within 3 days of the initial examination. In contrast with results of other studies, leukopenia and thrombocytopenia were uncommon in our study. Leukopenia (leukocyte count ≤4,000/μL) was present in 4 patients (33.3%) and thrombocytopenia (platelet count ≤150,000/μL) in 1 patient (8.3%). This finding may help distinguish CHIKV infection from dengue fever (*4*). Anemia (hemoglobin level ≤12 g/dL) was found in only 1 patient (8.3%). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination were available for 12 patients. ALT and AST levels were elevated (>40 IU/L) in 5 (41.7%) and 2 (16.7%) patients, respectively. Seven (46.7%) of 15 patients fully recovered within 1 month; 8 patients (53.3%) reported persistent arthralgia.

Because the GISPI network provides regional coverage only, the number of imported CHIKV cases in all of Italy in 2006 was likely higher. Moreover, most patients probably did not seek medical care, and when they did, physicians may have failed to recognize the disease because of lack of familiarity with it or limited diagnostic facilities. Differential diagnosis with other arthropodborne viruses of the *Alphavirus* genus (Ross River, Barmah Forest, o'nyong nyong, Sindbis, and Mayaro viruses) is difficult, but these are comparatively rare. In contrast, dengue and CHIKV epidemics may overlap, and potential patients should be screened for both.

The potential risk for introduction and establishment of CHIKV reservoirs in areas with mosquito vectors was discussed in March 2006 by a multidisciplinary European expert panel (*9*). In Italy, *A. albopictus* was first recorded in 1990; it has since quickly spread across the country. Scattered foci are now reported in almost all regions, mainly along the coastal plains, from the sea to the inlands, up to an altitude of ≈500–600 m (*7*).

The ability of *A. albopictus* to colonize new areas and its adaptability to the mild Italian climate allow vector populations to be active throughout the year (*10*). The patient is thought to be viremic for only 6–7 days (shortly before and during the febrile period) (*6*). We were unable to directly assess

Table. Demographic and epidemiologic data on 17 travelers with chikungunya infection diagnosed in 2006, Italy

Patient no.	Sex	Age, y	Reason for travel	Country of origin	Date of return (length of stay, d)	Date of first medical assessment after return (delay, d)	Last date of fever (length of fever, d)	Fever on date of return?
1*	M	32	Business	Réunion	Feb 23 (93)	Feb 25 (2)	Feb 26 (4)	Yes
2†	F	39	Tourism	Mauritius	Feb 28 (10)	Feb 28 (0)	Feb 28 (4)	Yes
3‡	M	46	Tourism	Mauritius	Mar 7 (10)	Mar 7 (0)	Mar 6 (5)	No
4‡	M	32	Tourism	Madagascar	Mar 7 (15)	Mar 8 (1)	Mar 4 (4)	No
5§	M	49	Tourism	Mauritius	Mar 08 (16)	Mar 15 (7)	Mar 4 (5)	No
6‡	M	66	Tourism	Madagascar	Mar 24 (15)	Mar 24 (0)	Mar 27 (5)	Yes
7§	M	36	Tourism	Mauritius	Apr 4 (15)	Apr 5 (1)	Apr 1 (6)	No
8*	F	43	Resident	Madagascar	Apr 10 (–)	Apr 11 (1)	Mar 2 (6)	No
9‡	F	46	Tourism	Réunion	Jan 30 (16)	Apr 13 (73)	NA (2)	–
10¶	F	44	Visit relatives	Mauritius	Apr 17 (33)	Apr 19 (2)	Apr 7 (12)	No
11‡	F	36	Tourism	Mauritius	Apr 30 (11)	May 3 (3)	May 3 (3)	Yes
12‡	M	31	Tourism	Réunion	Apr 21 (30)	May 4 (13)	Apr 5 (6)	No
13‡	M	44	Visit relatives	Cameroon	May 3 (24)	May 22 (19)	May 7 (6)	Yes
14*	M	35	Tourism	Seychelles	May 31 (9)	Jun 1 (1)	Jun 1 (2)	Yes
15*	M	38	Tourism	Mauritius	May 10 (11)	Jun 12 (2)	May 7 (4)	No
16‡	F	58	Missionary work	Central African Republic	Jun 24 (14 y)	Jul 10 (16)	Apr 26 (12)	No
17*	F	57	Business	India	Sep 8 (31)	Sep 9 (1)	Sep 10 (4)	Yes

*GISPI (Gruppo di Interesse e Studio delle Patologie di Importazione) centers: Torino.
†GISPI center: Udine.
‡GISPI center: Negrar. NA, not available.
§GISPI center: Brescia.
¶GISPI center: Triggiano.

viremia levels; however, almost half the patients were still febrile on return to Italy, which suggests a potential risk.

Although the same mosquito is a potential vector of dengue, no autochthonous case has been reported as yet, despite annual reports of many imported dengue cases in Italy. On the other hand, the clinical manifestations of both conditions are nonspecific, and a hypothetical autochthonous case would most likely go undiagnosed unless a targeted surveillance system were established. Prompt reporting of imported CHIKV infections is essential for monitoring of potential risk. The possibility of introducing CHIKV into Italy cannot be ruled out on the basis of current evidence.

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Dyella japonica Bacteremia in Hemodialysis Patient

To the Editor: Patients who receive long-term hemodialysis are at great risk for infection (*1*,*2*), especially bacteremia, which may lead to devastating outcomes (*3*). Environmental bacteria are commonly recovered from dialysis fluid, but their contribution to infection is less evident (*4*). We report a bacteremic episode caused by an unusual soil bacterium, *Dyella japonica.* The patient was a 69-year-old Thai woman who had had end-stage renal disease for 8 months and was receiving hemodialysis twice a week via subclavian double-lumen permanent catheter. Approximately 6 h after hemodialysis, she became febrile. Physical examination showed temperature 38°C, respiratory rate 22/min, heart rate 80/min, and blood pressure 130/60 mmHg. The rest of her examination was unremarkable and included normal state of consciousness, clear eyeground (fundus), and absence of a heart murmur. Her catheter was intact without evidence of exit site or catheter infection.

Two blood samples, 1 each from the central line and peripheral line, were injected into BACTEC Aerobic/F bottles and incubated in the BACTEC 9240 system (Becton-Dickinson Diagnostic Systems, Sparks, MD, USA). A catheter-related bacteremia was suspected, and vancomycin (1 g in intravenous drip) was prescribed. Other laboratory findings included a total leukocyte count 14.5 × 10^9/L (84% neutrophils, 16% lymphocytes), blood urea nitrogen 38 mg/dL, and creatinine 7.9 mg/dL. Urinalysis results were within normal limits. Urine and stool cultures were negative for pathogenic bacteria. The catheter was not removed for culture. On day 4 of incubation, both blood cultures showed growth, which was then placed onto 5% (vol/vol) sheep blood agar for subculture and produced deep yellow colonies. This uniform, gram-negative, oxidase-positive bacterium was not identifiable with manual phenotypic tests and the API 20NE strip (bioMérieux, Durham, NC, USA). It was identified by the Vitek 2 system (bioMérieux) and reported to be *Myroides* sp. with an excellent confidence level (98.7% probability).

To further confirm the identification, we used 16S rDNA analysis. The primer pair forward 5′-AGAGTTTGATCMTGGCTCAG-3′ and reverse 5′-ACGGYTACCTTGTTACGACTT-3′ were used to amplify the 16S rDNA by PCR. DNA extraction and PCR amplification were carried out as described (*5*). The sequence of 16S rDNA amplicon (1,450 bp) was determined after electrophoresis and performed with the 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. The 16S rDNA sequence of this isolate (strain RB28), deposited in GenBank under accession no. DQ984127, was compared with sequences in GenBank by using the BLAST algorithm (version 2.0; National Center for Biotechnology Information, Bethesda, MD, USA, www.ncbi.nlm.nih.gov/blast). Sequence alignment and distance analysis were performed with Lasergene software (DNASTAR, Inc., Madison, WI, USA). According to the 16S rDNA sequence analysis, our isolate belonged to the family *Xanthomonadaceae* of the Gamma Proteobacteria class; the highest sequence similarity (99.2%) was obtained for *D. japonica* type strain XD53 (*6*). In contrast, RB28 shared <97% sequence similarity to other species of *Dyella* and other genera in this family (data not shown). Organisms within the same species should share ≥97% of 16S rDNA sequence similarity (*7*). Therefore, this isolate was identified as *D. japonica.* The biochemical profile of RB28 was also most consistent with *D. japonica* (Table).

MIC values as determined by Etest were amikacin 0.75, cefotaxime 0.064, ceftazidime 0.38, ciprofloxacin <0.002, co-trimoxazole 0.125, gentamicin 1.5, imipenem and meropenem 0.25 mg/L. Because of MIC results, treatment was changed to ceftazidime (1 g intravenously every 8 h). Fever abated within a few days without catheter removal. The patient had a complete recovery with no complications. Follow-up blood cultures 2 and 4 weeks after 14 days of treatment were negative.

The *Dyella* genus comprises 3 species: *D. japonica* (*6*), *D. koreensis* (*8*), and *D. yeojuensis* (*9*). All are soil isolates and have been neither isolated from clinical samples nor reported to cause human infection. Their pathogenicity in humans is unknown. Because of its rapid onset after hemodialysis, the bacteremia in this patient is thought to have been associated with the dialysis procedures. Contaminated dialyzing fluid may have been a source for the organism, and the permanent catheter was likely to have provided an entry. In addition, blood culture bottles could have been contaminated by environmental samples. However, the diagnosis of catheter-related infection could not be definitive because neither catheter tip nor fluid was available for culture. The severity of *D. japonica* bacteremia was difficult to determine because the clinical manifestation was

Table. Biochemical characteristics of patient's isolate RB28 and type strains of *Dyella* species*

Characteristics	Patient's isolate RB28	*D. japonica* XD53^T	*D. koreensis* BB4^T
Oxidase	+	+	+
Catalase	+	+	w
Motility	+	+	+
Acid from			
L-arabinose	–	–	–
D-galactose	–	–	–
D-glucose	+	+	+
D-mannose	+	+	–
D-ribose	–	–	–
D-sucrose	–	–	+
D-xylose	–	–	–
Caprate	–	–	–
Citrate	–	–	–
α-galactosidase	–	–	+
β-*N*-acetyl-glucosaminidase	–	w	+
α-glucosidase	–	w	+

*Data from references (*6*) and (*8*); T, type strain.

mild and the patient responded well to antimicrobial drug therapy, albeit without catheter removal. This case emphasizes that environmental bacteria can be an emerging threat for hemodialysis patients, who are at risk of acquiring opportunistic infection. In addition, this report demonstrates the usefulness of molecular methods for identifying uncommon isolates.

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Mycobacterium cosmeticum, Ohio and Venezuela

To the Editor: *Mycobacterium cosmeticum* is a rapidly growing nontuberculous mycobacteria species that was first described in November 2004. The first strains were obtained from cultures of a sink drain in a nail salon in Atlanta, Georgia, USA, and from a granulomatous lesion of a female mesotherapy patient in Venezuela (*1*).

Among 3 additional isolates of *M. cosmeticum* obtained from July 2003 through November 2004, one was obtained from a 77-year-old man who was admitted to Ohio hospital A on September 22, 2004, with fever, exacerbation of chronic obstructive pulmonary disease, and urosepsis. Underlying medical conditions included diabetes, discitis, hyperlipidemia, coronary artery disease, and coal worker's pneumoconiosis. He had received intravenous antimicrobial agents (rifampin and daptomycin) through a Groshong catheter that had been inserted to treat discitis. A routine blood culture was performed according to standard methods (*2*), and the catheter was removed. A diagnosis of catheter-associated bacteremia (CAB) was made, but the patient's overall condition improved without antibacterial drug therapy, and he was discharged 4 days after admission. The culture specimen yielded only mycobacteria and was sent on to ARUP Laboratories, where it was identified as *M. cosmeticum* by 16S rDNA sequence analysis. The isolate was then sent to the Centers for Disease Control and Prevention (CDC) Mycobacteriology Laboratory Branch (Atlanta, GA, USA) and designated OH1.

A 43-year-old woman with a diagnosis of non-Hodgkin lymphoma, who had received regular central venous catheterizations, was admitted to Ohio hospital B on August 20, 2004. A left subclavian catheter was inserted, and

a routine blood specimen for culture was subsequently obtained on the day of admission. Before admission, the woman had been receiving acyclovir and cefepime. She received chemotherapeutic agent injections, platelet infusion, and an autologous stem cell transplant 6 days after admission. The blood culture was positive only for rapidly growing mycobacteria, and the final diagnosis was CAB. However, no symptoms of infection were observed, and no antimycobacterial drug therapy was administered. She was discharged without complications after the transplant was received and the catheter removed. The bacterial isolate was forwarded to CDC's Special Bacteriology Reference Laboratory, where it was identified as *M. cosmeticum* by 16S rDNA sequence analysis, sent on to the CDC Mycobacteriology Laboratory Branch, and designated OH2.

A 36-year-old man with AIDS was admitted to hospital C in Caracas, Venezuela, in June 2003 with dyspnea, fever, and expectoration. A sputum sample was positive by acid-fast bacillus smear and culture, yielding both *M. cosmeticum* (designated VZ1) and *M. scrofulaceum* on Middlebrook 7H10 agar (Remel Co., Lenexa, KS, USA). At the time the sputum was obtained, the patient was receiving only trimethoprim-sulfamethoxazole, but he experienced respiratory arrest and died ≈6 weeks later.

The 3 isolates were confirmed to be *M. cosmeticum* by high-performance liquid chromatography mycolate analyses and by PCR restriction analysis of a 440-bp segment of *hsp*65 (*1*). The relationship of these isolates to the only documented strains of *M. cosmeticum* was evaluated by analysis of large restriction fragments with pulsed-field gel electrophoresis (*1*) and by repetitive element PCR (*3*). Banding patterns for isolates OH1 and OH2 were different from one another as well as from isolate VZ1 and the 2 control strains. Typing patterns for isolate VZ1, however, matched the control strain from Venezuela (ATCC BAA-878^T), which indicates that these 2 isolates are likely a common strain (Figure).

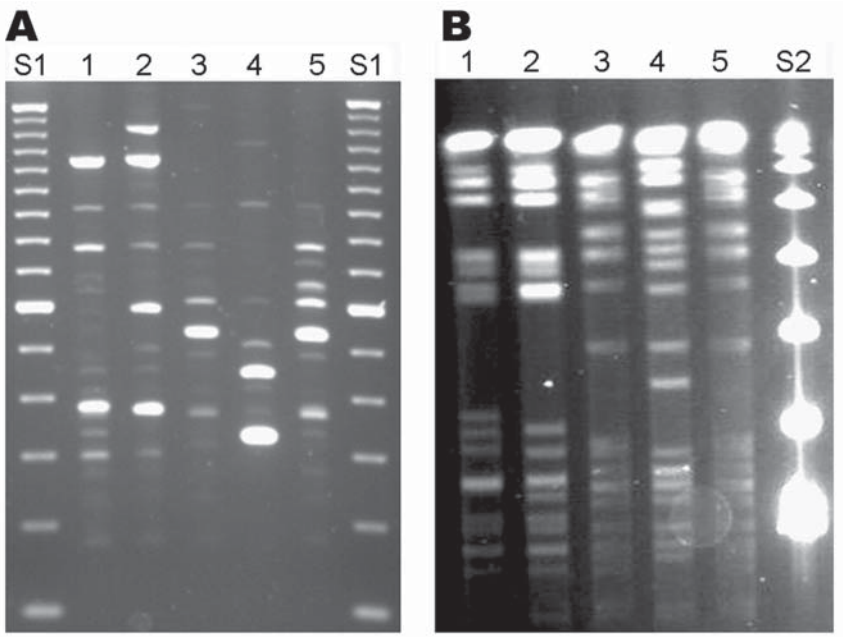

Figure. Repetitive element (Rep)–PCR (A) and pulsed-field gel electrophoresis (PFGE) (B) patterns of *Mycobacterium cosmeticum* isolates from 2 patients in Ohio and 1 patient in Venezuela. Rep-PCR was performed by using BOXA1R primer (*3*), and PFGE was performed with restriction enzyme *Ase*I. Lanes 1, 2, Ohio isolates OH1 and OH2; lanes 3, 4, control strains ATCC BAA-878^T and ATCC BAA-879; lane 5, Venezuelan isolate VZ1. DNA size standards are 100-bp (S1) and 48.5-kb marker (S2).

Of the >125 recognized *Mycobacterium* species, ≈50 are etiologic agents of human disease (*4*). The type strain of *M. cosmeticum* (ATCC BAA-878^T) was associated with a soft-tissue infection in which the source was postulated to be environmental contamination of an unknown substance administered to the patient by injection as part of a weight loss regimen. This strain and isolate VZ1 were isolated from clinics in Caracas, Venezuela; both were found to be a common strain, but no other factors suggest that these represent an epidemic cluster. Although the Venezuelan patient from whom isolate VZ1 was obtained exhibited symptoms consistent with mycobacterial pulmonary disease, *M. cosmeticum* involvement cannot be proven because an additional *Mycobacterium* species, *M. scrofulaceum*, was isolated from the patient's sputum. Because each of these organisms is found in the aqueous environment, they may represent colonization or may have been transiently present in the patient. Nonetheless, additional nontuberculous mycobacteria species have been reported to cause pulmonary disease, and the involvement of *M. cosmeticum* in this case cannot be excluded.

Successful treatment of CAB infections caused by rapidly growing mycobacteria has most often been achieved by removing the catheter with or without the use of antimicrobial drug therapy (*4*). Criteria to support a true bloodstream infection were met by one of the patients in Ohio. These criteria include the absence of a source for bacteremia alternative to *M. cosmeticum* OH1 and the resolution of the febrile syndrome after removal of the device. The second patient had no symptoms when the blood culture was obtained; thus, the clinical significance of *M. cosmeticum* in this case is unclear.

When all identified strains of *M. cosmeticum* are considered, this species is clearly present in diverse geographic regions and in healthcare institutions. These findings suggest that it may be widely distributed in the environment and should be regarded, along with other rapidly growing mycobacteria species, as a potential pathogen.

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Ecoregional Dominance in Spatial Distribution of Avian Influenza (H5N1) Outbreaks

To the Editor: Recent articles in Emerging Infectious Diseases (*1*,*2*) and elsewhere (*3*,*4*) have highlighted the role of Anatidae migration in dispersal of the H5N1 subtype of highly pathogenic avian influenza (HPAI) virus. Although these articles point out that identifying the geographic origin of migrating waterfowl is needed to understand and predict pathogen dispersal, study analyses have been limited to pathways with nominal reference to climatic and vegetation patterns that control spatiotemporal patterns of this migration.

We propose that a better understanding of the threat of future spread can be obtained by identifying specific climatic and vegetation zones that are important in the life cycle of Anatidae, and which account for a disproportionately large number of HPAI outbreaks. The concept of ecoregions (*5*,*6*), i.e., distinct assemblages of natural communities determined by climate, geology, and evolution, is a useful zonal classification for evaluating HPAI outbreaks. A World Wildlife Fund classification delineating 825 terrestrial ecoregions (*7*), combined with a Google Earth map of 3,133 avian influenza outbreaks from November 24, 2003, to November 21, 2006 (*8*), provided the basis for this analysis. All files were converted to shapefiles (Environmental Systems Research Institute, Redlands, CA, USA), and overlay analysis was performed by using ArcGIS software (Environmental Systems Research Institute).

The online Appendix Figure (available from www.cdc.gov/EID/content/13/8/1269-appG.htm) shows a chloropleth map (display of quantitative or qualitative information about subentities in terms of symbols or colors) of ecoregions with numbers of avian influenza cases (each spatially and temporally isolated set of individual events, regardless of number of deaths, is recorded as a case). Panels A, B, and C of this figure show enlargements of specific ecoregions with large numbers of known cases in regions of Eurasia, Southeast Asia, and Africa, respectively. Twenty-five ecoregions, representing 8.8% of the terrestrial surface area, accounted for 2,407 (76.8%) cases. A total of 132 of 825 ecoregional classifications had ≥1 recorded case of an avian influenza outbreak, but most (83) had <10 cases each.

Regionally, Southeast Asia has 12 ecoregions that collectively account for 1,651 cases (online Appendix Figure, panel B) that have occurred consistently, albeit cyclically, since 2003. Among these ecoregions, the freshwater wetlands of the Chao Phraya, Tonle Sap, and Red Rivers are known migratory waterfowl wintering habitats in which 719 cases were located. Recent phylogenetic evidence suggests that this area is a local hotspot for an endemic strain of avian influenza (H5N1) that demonstrates bidirectional dispersal among localities within the region (*9*).

In the Eurasian region (online Appendix Figure, panel A), 12 ecoregions accounted for 712 cases. The easternmost ecoregions, the Kazakh forest steppe (location of Lake Chany, an Anatidae habitat and breeding area) and the Kazakh Steppe, accounted for 132 cases, with the first case recorded

on July 18, 2005. Subsequent major outbreaks in this region occurred in July–August 2005 and December 2005–January 2006. Regions around the Black Sea, including Euxine-Colchic broadleaf forests (deltas of the Kizil and Yesil Rivers), westernmost Pontic steppe (Lake Sivash), and Balkan mixed forests (deltas of the Danube, Olt, and Siret Rivers) have been loci for outbreaks in the central Eurasian region since October 1, 2005. Additional outbreaks have occurred since October 21, 2005, in the Eastern Anatolia montane steppe and deciduous forests (location of Lakes Van and Urmia, and Karakaya and Keban Baraji Reservoirs). Mixed and broadleaf forests of central and western Europe account for the remaining European cases since October 19, 2005. Anatidae habitats in this area include freshwater wetlands formed by the Danube, Rhine, Rhone, and Saone Rivers, and the Baltic basin.

Two African ecoregions, the Nile Delta–flooded savanna (online Appendix Figure, panel A) and the West Sudanian savanna (online Appendix Figure, panel C), including part of the Lake Chad ecosystem, i.e., the Kano River and the Tiga Reservoir, accounted for 79 cases. The initial Sudanian savanna case was identified on January 10, 2006, and the initial Nile Delta case was identified on February 17, 2006.

Our results may be skewed by several confounding factors, e.g., low national surveillance capabilities resulting in unreported cases and effects of the poultry trade. Nonetheless, the findings have implications for global monitoring of avian influenza (H5N1) outbreaks. Although migratory pathways and the poultry trade should continue to be scrutinized, monitoring efforts should focus on wintering and breeding habitats of migrating waterfowl, especially wetlands located within ecoregions with a disproportionately large number of avian influenza outbreaks. These hotspots are also likely to give rise to endemic local strains with regional dispersal characteristics (*9*).

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In Response: Ecoregions are large units of land that reflect the distribution of species and communities and are used to define priority areas for biodiversity conservation. In their letter (*1*), Sengupta et al. suggest that ecoregions could help explain the spatial distribution of observed outbreaks of HPAI (H5N1). Since most outbreaks they observed occurred in ecoregions with waterfowl habitats, they concluded that monitoring efforts should focus on these areas.

Habitat mapping is useful for predicting the spread of wildlife-associated diseases. However, because ecoregions are large areas where agriculture and other human activities abound, many confounding variables exist. To assume that ecoregions with HPAI (H5N1) outbreaks are chiefly characterized by the natural biotopes is too simplistic.

In addition, the authors assume that wild waterfowl are the main factors in the dispersal of HPAI (H5N1). Although wild birds are known to be involved in the dispersal of low pathogenic strains of avian influenza viruses (*2*,*3*), the precise role of these birds in the dispersal of HPAI (H5N1) viruses remains controversial. It is now clear that the expansion of HPAI (H5N1) in Asia and to Africa was primarily associated with the poultry trade (*4*), which is not represented on a map of ecoregions. Also noteworthy is that HPAI (H5N1) did not persist in wild birds after the spring of 2006, and no case has been detected in the wetlands of America despite the migration of wild ducks and waders from Siberia (their main breeding ground) to Asia, Europe, and North America.

We believe that human activity is the primary vehicle for the spread of HPAI (H5N1) virus. Rather than the monitoring of waterfowl habitats, increased surveillance of the legal and illegal trade of birds and bird products is more likely to help in predicting the spread of this virus.

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In Response: Sengupta et al. (*1*) discuss the role of ecoregions in the distribution of HPAI (H5N1) outbreaks. Although the concept of ecoregions is undoubtedly useful in global biogeography, we do not understand the point they are trying to make. In our article (*2*), which is cited in their letter, we undertook a descriptive study to determine whether spread of HPAI (H5N1) virus was consistent in time with ecologic drivers of bird migration and in space with distribution of major migratory flyways of Anatidae. It is obvious that the distribution pattern of Anatidae is dependent on ecologic variables, and some of these variables are summarized by the ecoregion concept.

However, apart from a strictly descriptive point of view, we do not see how the ecoregion concept applies to describe patterns in HPAI (H5N1) spread and distribution. Sengupta et al. list ecoregions where reports of HPAI (H5N1) were concentrated. However, what do they infer from this? They observe regions with many reports of HPAI (H5N1) and conclude that these ecoregions are at risk. We find this reasoning completely circular, and any geographic zonation would provide the same observation. They may mean that ecoregions define boundaries within which secondary spread of HPAI is more likely than across ecoregions. However, this hypothesis would need to be more clearly demonstrated and quantified before the ecoregion concept can be used for global monitoring of HPAI (H5N1) outbreaks.

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Nephropathia Epidemica in Metropolitan Area, Germany

To the Editor: Old World hantaviruses (family *Bunyaviridae*) are rodentborne pathogens that can cause hemorrhagic fever with renal syndrome (HFRS) (*1*). At least 3 different pathogenic hantavirus species have been detected in Europe: *Dobrava-Belgrade virus* (DOBV), *Tula virus,* and *Puumala virus* (PUUV) (*1–3*). Most human hantavirus infections in Europe are assigned to PUUV transmitted by bank voles (*Myodes glareolus*, formerly *Clethrionomys glareolus*). Although PUUV is thought to cause a mild form of HFRS, designated as nephropathia epidemica (NE), severe courses have been described with a case-fatality ratio of up to 0.6% (*3*).

Even though human hantavirus infections have sporadically been reported in Germany since 1983 (e.g., *4–7*), clinically apparent hantavirus infections (HFRS, NE) did not become notifiable diseases in Germany until 2001. From 2001 through 2004, ≈140 to 240 cases per year have officially been documented in Germany; most were caused by PUUV. Regions endemic for PUUV have been identified in southern Germany, especially the Alb-Danube region (*4*,*6*,*8*). Since 2004, 2 aspects of the situation in Germany have changed. First, the number of clinical cases has increased dramatically to a total of 448 in 2005. Second, hantavirus infections have been observed in regions previously not recognized as endemic for hantaviruses (*9*). An increased number of human cases were also observed in other European countries (*10*).

Here we report the first, to our knowledge, documented PUUV-associated urban NE outbreak, which occurred in a city park in Germany. In 2005, a total of 89 cases were reported in the district of Cologne with

41 cases recorded in the city center (incidence 4.2/100 000). In the past, (2001–2004), 3–22 cases were reported annually for the district of Cologne and 2–6 cases for the city of Cologne. Clinical symptoms, documented by responses to a questionnaire, resembled those typical for NE found in previous studies in Germany (*4*,*5*,*7*,*8*) and included fever (93%), headache (43%), and arthralgia (40%), without hemorrhage. Renal dysfunction was found in ≈83% of patients, and approximately three-fourths of the patients were temporarily hospitalized (n = 29). Serologic investigations by ELISA and indirect immunofluorescence assay confirmed PUUV-reactive immunoglobulin M (IgM) and IgG antibodies in serum specimens from all 89 patients. The average age of the patients was 39 years (range 6–65 years), and the male/female ratio was 2.6:1.

For a large number of patients, the exposure to PUUV most likely occurred in a forested park and recreation area ("Stadtwald," 20 ha) in Cologne's inner city circle (Figure) where they lived, worked, or enjoyed recreational activities. Five patients had homes adjacent to this area. Four patients were evaluated for likely exposure due to employment at the RheinEnergie Stadium, 1 player in the German Football League and 3 employees who had cleaned basements or attics at the stadium (Figure). Three patients were members of a tennis club located near the stadium. To further investigate these cases, in April and June 2005, rodents were trapped in the Stadtwald. The effort yielded 35 bank voles, 17 yellow-necked mice, and 1 wood mouse. Screening of 48 available serum specimens by ELISA with yeast-expressed nucleocapsid (N) proteins of PUUV and DOBV (*9*) demonstrated 19 reactive blood samples. Seventeen had a higher endpoint titer to PUUV, and 2 showed identical endpoint titers for PUUV and DOBV. These 19 reactive samples (63%) originated from 30 *M. glareolus* bank voles.

Lung tissues of all 53 mice were analyzed by PUUV-specific reverse transcription (RT)–PCRs targeting the S segment (for primers, see [*9*]). In 23 (66%) of the 35 bank voles, but in none of the other rodents, PUUV-specific RNA was amplified and sequenced. The concordance of ELISA– and RT-PCR–positive samples was 98% (online Appendix Table, available from www.cdc.gov/EID/content/13/8/1271-appT.htm).

Figure. City of Cologne, showing its corridor of wooded public parks (shaded area) and the location of the exposure sites in the Stadtwald stadium area: 1, Cologne cathedral; 2, Stadtwald; 3, RheinEnergie Stadium; 4, university; 5, trade fair; 6, airport.

Comparison of the partial S-segment nucleotide sequences obtained showed intersequence distances of 0%–1.2%. The level of the nucleotide sequence divergence from previously described German PUUV strains was 14.7%–16%. In phylogenetic analyses (neighbor-joining, maximum likelihood) based on this fragment, all sequences from the Cologne cluster formed a distinct group next to the branch consisting of strain Erft (95.4%–96% identical). These strains were clearly separated from additional PUUV strains originating from Germany (Berkel, southeastern Germany, Heidelberg), and Belgium, a neighboring European country (data not shown).

The only hantavirus known in an urban environment is Seoul virus, which is transmitted mainly by the peridomestic brown rat (*Rattus norvegicus*). As with most other hantaviruses, PUUV patients, including those previously observed in Germany, were reported to become infected in their rural residences or, when living in urban regions, during visits to the countryside in their spare time (*4*).

To our knowledge, this is the first report describing an outbreak of PUUV infections in a metropolitan area in Europe with a defined exposure site in the city center. The PUUV outbreak in 1990 in the city of Ulm occurred due to exposure during field military maneuvers in the outskirts of the city near the Danube River, and the surrounding civilian population did not experience a similar outbreak (*6*). The exposure site of a previous cluster of PUUV-infected patients reported from the city of Ulm and its surroundings remain uncertain but might be also rural because almost all patients lived outside

the city of Ulm (*8*). In Cologne, about two thirds of the bank voles captured at the exposure site carried PUUV and are assumed to be the most probable source of infection. Increased sightings of rodents were reported by local health offices and pest control units. Studies at putative exposure sites in southeastern Germany in 2004 also showed a high prevalence of PUUV in the respective bank vole populations.

These cases are also the first indication, to our knowledge, that recreational activities in a forested city park, infested by hantavirus-infected rodents, may lead to human infections. This possibility should be investigated carefully in outbreak situations and may have practical implications for the future surveillance and prevention of NE in Europe.

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Effect of Hurricane Katrina on Arboviral Disease Transmission

To the Editor: Rarely has the aftermath of a natural disaster in the continental United States resulted in increased transmission of mosquito-borne viruses (*1*). However, on August 29, 2005, Hurricane Katrina struck Louisiana and Mississippi, where mosquito-borne West Nile (WNV) and St. Louis encephalitis viruses are endemic.

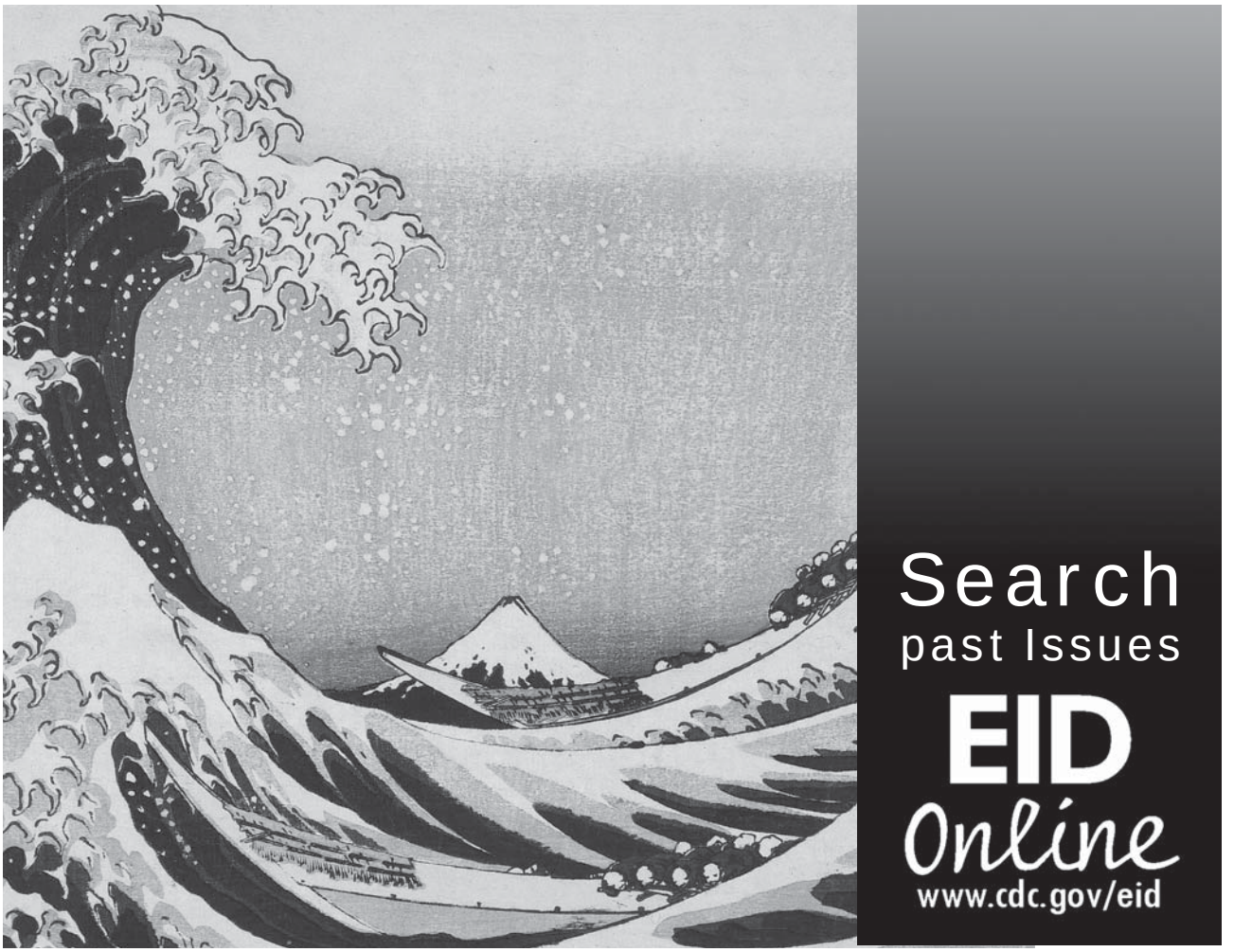
Using data from the ArboNET system of the Centers for Disease Control and Prevention, we evaluated the short-term effects of Hurricane Katrina on the reported incidence of human West Nile neuroinvasive disease (WNND) and Saint Louis encephalitis (SLE) in Louisiana and Mississippi using the reported week of onset and the year (2003–2005). We also evaluated incidence by onset date and county (or parish) over 3 time intervals (January 1–August 31, September 1–September 30, and October 1–October 30) in 2005. Reporting lag was evaluated by onset dates and corresponding dates of reports. Because the completeness of reporting of West Nile fever and other arboviral fever cases is highly variable, only reports of human WNND and SLE were considered.

In Louisiana, the highest reported incidence of WNND occurred in the second week of August 2005, before Hurricane Katrina made landfall. Al-

though the number of cases reported in 2005 (117) was higher than in 2003 (85) or 2004 (101), the number of cases peaked during roughly the same weeks in each year. In Mississippi, the total number of cases reported in 2005 (39) was only slightly higher than in previous years (31 in 2004 and 34 in 2003). The number of cases peaked in mid-September 2005, later than the peak in 2004, but similar to when a second peak occurred in 2003. Thus, the increase in WNND incidence for either state does not appear to be hurricane-related.

In Louisiana, 82 WNND cases in 20 parishes had onset between January 1, 2005 and August 31, 2005. In comparison, 25 WNND cases had onset between September 1, 2005 and September 30, 2005; a total of 14 of these cases in 7 parishes had not reported WNND cases previously in 2005; a total of 5 of these parishes had detected WNV activity in animals before the hurricane. From October 1, 2005 to October 31, 2005, a total of 10 additional WNND cases were reported in Louisiana, including 1 case from a parish that had not previously reported cases. Only 5 cases with illness onset after the hurricane resided in coastal parishes. In Mississippi, 17 WNND cases in 10 counties had onset between January 1, 2005 and August 31, 2005. Twenty cases had onset between September 1, 2005 and September 30, 2005, including 9 cases in 4 counties that had not reported WNND cases previously in 2005; a total of 2 of these counties had detected WNV activity in animals before the hurricane. From October 1, 2005 to October 31, 2005 2 more WNND cases were reported in Mississippi, including 1 from a county that had not reported cases previously. All cases with illness onset after the hurricane resided in inland counties. Thus, in both states the coastal counties and parishes that were hardest hit by the hurricane had the fewest number of posthurricane WNND cases.

In 2005, Louisiana reported 2 SLE cases and Mississippi reported 5. Both Louisiana cases and 4 of the Mississippi cases had onset of illness after September 1. In 2004, no SLE cases had been reported by either of these states. In 2003, a total of 9 cases were reported in Louisiana (3 with onset in September) and 2 in Mississippi (with onsets in May and June). Thus, Hurricane Katrina did not appear to



increase SLE incidence in Louisiana, and if it did increase incidence in Mississippi, the increase was minimal.

In both 2003 and 2004, Louisiana's median reporting time to ArboNET was ≈30 days. In 2005, the median reporting time prehurricane was 36 days and posthurricane was 69 days. Louisiana state officials believed that this reporting lag was largely due to impaired transport and collection of biologic samples and relocation of diagnostic facilities immediately following the hurricane. In contrast, in 2003 and 2004, Mississippi's median reporting time to ArboNET was 21 days and 36 days, respectively. In 2005, the median reporting time prehurricane was 23 days and posthurricane was 14 days. Mississippi state officials believed that the improved reporting time was due to the additional help and longer hours worked by health department officials following the hurricane.

Although Hurricane Katrina disrupted WNV surveillance in Louisiana, it did not appear to increase the incidence of WNND and SLE in either Louisiana or Mississippi. In coastal areas, the hurricane destroyed housing and impeded vector control, thus possibly increasing the risk of mosquito-borne infections (*1*,*2*). However, hurricane-force winds and heavy flooding might have actually decreased the risk of WNV and SLE transmission by dispersing or killing birds and mosquitoes, and destroying their habitat. Many people were promptly evacuated to less affected areas, where, on the basis of previous years' data showing seasonality of WNV transmission, the risk of infection was probably decreasing. Natural disasters do not usually cause an immediate increase in arboviral diseases (*1*,*2*). However, if hurricanes strike early in transmission season, there could be a late increase in risk after vector and host populations are re-established. In addition, risk could increase when people are relocated to areas where transmission is intense.

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Threat to Cefixime Treatment for Gonorrhea

To the Editor: From November 2002 through May 2003, a total of 4 Japanese men, ranging in age from 23 to 45 years, visited the Department of Urology at Toyota Memorial Hospital, Toyota, Japan. Physical examinations showed urethral discharge and dysuria. Each had had sexual contact with sex workers in central Japan. Four strains of *Neisseria gonorrhoeae* were isolated from urethral specimens. Treatment comprised 200 mg cefixime, twice a day for 3 days. However, all 4 patients returned to the clinic with continuing symptoms, despite having completed the prescribed course of cefixime and abstaining from sexual activity. *N. gonorrhoeae* was again isolated from urethral swabs. Each patient was then treated with 1 g intravenous ceftriaxone. In the 3 patients who returned to the clinic for followup, the ceftriaxone treatment resulted in clinical and microbiologic cure.

Pulsed-field gel electrophoresis (PFGE) analysis of the *Spe*I-digested DNAs of *N. gonorrhoeae* was performed to assess the relatedness of pre- and posttreatment isolates (*1*). For these 8 isolates, MICs of penicillin G, tetracycline, cefixime, cefdinir, cefodizime, ceftriaxone, levofloxacin, azithromycin, and spectinomycin were determined on chocolate agar (GC) medium base supplemented with 1% IsoVital X (Becton Dickinson, Franklin Lakes, NJ, USA) and containing serial 2-fold dilutions of each agent (*2*). Media were inoculated with 10^4 CFU and incubated at 35°C in 5% CO_2 overnight. The MIC was defined as the lowest concentration inhibiting growth to ≤1 CFU. β-lactamase activity of the isolates was tested with a nitrocefin disk. The nucleotide sequences of the full-length *penA* gene encoding the penicillin-binding protein 2 (PBP 2) were identified in the isolates (*1*). Briefly, genomic DNAs from each isolate were subjected to PCR to amplify 3 fragments of the *penA* gene of *N. gonorrrhoeae*. PCR products were sequenced by the dye terminator method and with an automatic sequencer.

In each of the 4 cases, the PFGE patterns of the pre- and posttreatment isolates had the same numbers of bands (12–16 fragments), and the corresponding bands were the same apparent size; the pre- and posttreatment isolates were indistinguishable (*3*). MICs of antimicrobial agents for the 8 isolates are shown in the Table. All isolates were enzymatically negative for β-lactamase and possessed identical mosaic alterations in PBP 2. The mosaic PBP 2 was composed of fragments of PBP 2 from *N. cinerea* and *N. perflava* and was identical to that identified in our previous study (*1*).

Until recently, Japanese guidelines recommended oral administration of cefixime, 200 mg twice a day

Table. Antimicrobial drug susceptibilities of clinical isolates of *Neisseria gonorrhoeae* from patients with gonococcal urethritis treated unsuccessfully with a 3-day cefixime regimen*

		MIC (μg/mL)								
Patient	Isolate	PCG	TET	CFX	CFD	CDZ	CTX	LVF	AZM	SPC
1	Pre-Tx	4	2	0.5	1	0.125	0.125	16	0.25	16
	Post-Tx	4	2	0.5	1	0.125	0.125	16	0.125	32
2	Pre-Tx	8	4	0.5	1	0.25	0.5	8	0.5	32
	Post-Tx	8	2	0.5	1	0.125	0.25	8	0.5	16
3	Pre-Tx	4	2	1	2	0.25	0.125	16	0.25	16
	Post-Tx	4	2	1	2	0.25	0.125	16	0.25	16
4	Pre-Tx	4	1	1	2	0.25	0.25	16	0.125	16
	Post-Tx	4	2	1	2	0.25	0.25	16	0.25	32

*PCG, penicillin G; TET, tetracycline; CFX, cefixime; CFD, cefdinir; CDZ, cefodizime; CTX, ceftriaxone; LVF, levofloxacin; AZM, azithromycin; SPC, spectinomycin; Pre-Tx, isolate recovered before cefixime treatment; Post-Tx, isolate recovered after cefixime treatment, i.e., before ceftriaxone treatment.

for 3 days to prolong the period of time for which the serum drug concentration remains above the MIC (*4*). However, treatment failure with this cefixime regimen was observed in our 4 cases of gonorrhea. The isolates showed cefixime MICs of 0.5–1 μg/mL and harbored mosaic alterations in PBP 2. Most recently, the emergence and spread of such strains in Japan (*1*,*5*,*6*) have led to the recommendation that ceftriaxone and spectinomycin should be used as the primary therapy for gonorrhea instead of oral cephalosporins (*7*).

In 2001, treatment failure was reported in a Caucasian man residing in Hawaii who had been given a single 400-mg dose of cefixime for gonorrhea (*8*). Pre- and posttreatment strains of *N. gonorrhoeae* were recovered from this patient, and 1 strain was isolated from his Japanese female sex partner who had visited Hawaii from Japan. Another pretreatment strain was isolated from a Micronesian man with gonorrhea residing in Hawaii, who had had sex with a woman from Malaysia or the Marshall Islands. This man was successfully treated with a single 400-mg dose of cefixime. For these strains, MICs of penicillin, tetracycline, spectinomycin, cefixime, ceftriaxone, ciprofloxacin, and azithromycin were 8.0, 4.0–8.0, ≤32, 0.25–0.5, 0.125, 8–16, and 0.125–0.25 μg/mL, respectively. The antibiograms of these strains in Hawaii were similar to those of strains with mosaic PBP 2 found in our 4 patients. The introduction to Hawaii of such multidrug resistant strains might be related to sex partners from Asia. Although the strains were not analyzed for alterations in PBP 2, they could have been derived from strains with cefixime resistance-associated mosaic PBP 2.

The strains with mosaic PBP 2 showed such decreased susceptibility to cefixime that they were not effectively eradicated by the 3-day treatment. Although it is not clear whether these strains are also resistant to the single 400-mg dose of cefixime (*8*, *9*), their emergence and spread could threaten treatment for gonorrhea with cefixime (*10*). Global emergence and spread of such multidrug-resistant strains of *N. gonorrhoeae* would be a matter of serious concern. The antimicrobial susceptibilities of current gonococcal isolates must be monitored periodically. In particular, posttreatment isolates from patients treated unsuccessfully with cefixime should be surveyed.

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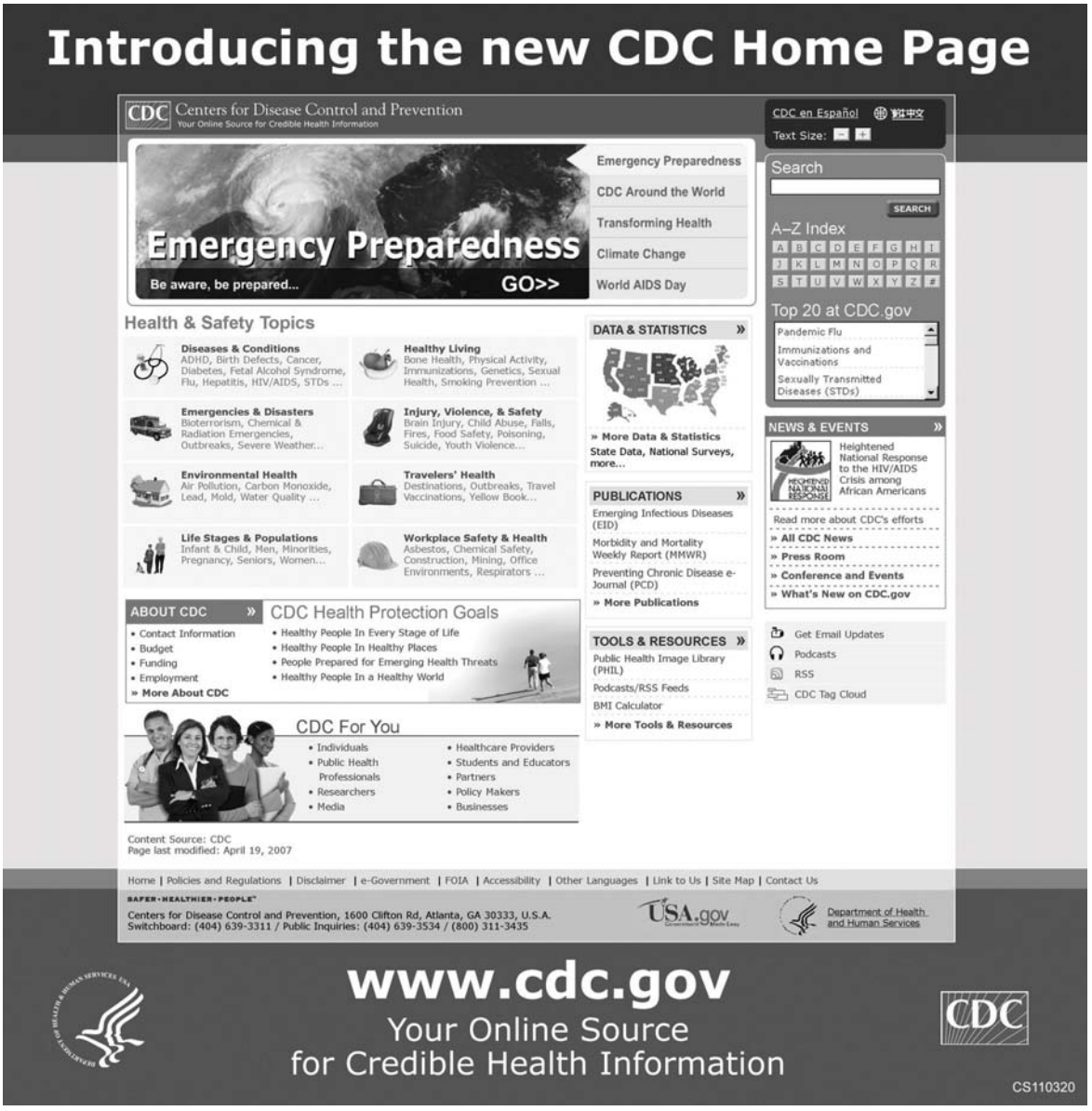

Vector- and Rodent-borne Diseases in Europe and North America: Distribution, Public Health Burden and Control

Norman Gratz

Cambridge University Press, New York, New York, USA, 2006
ISBN: 0-521-85447-4
Pages: 393; Price: US $120.00

An estimated 70% or more of emerging infectious disease agents have some form of vector. The author of this book, Norman Gratz, a medical entomologist, has written a very valuable resource on vectorborne and rodentborne diseases found in Europe and North America. His book includes information on diseases transmitted by mosquitoes, ticks, rodents, mites, sandflies, fleas, lice, biting midges, diptera, triatomines, and cockroaches. As a text, it is impressive that one man has such a breadth of knowledge of these diseases, although some credit must go to Mike Service, who helped prepare the manuscript for publication after the death of the author.

Unlike most books on this subject, which concentrate on describing the agent and its molecular properties, this book focuses on public health aspects of the diseases, and most chapters are divided on the basis of the vector that carries the agents. The chapters describe the history of the agents and details of incidence by country, year, and important ecologic parameters. The details of the number of cases of each disease by year are particularly impressive. Many chapters have a conclusion section with an overview of the public health importance of the disease, interpretation of the risk of the disease, and identification of our knowledge gaps. In addition, some chapters are devoted to the economic effects of vectorborne and rodentborne diseases in Europe and North America.

This is a very readable text or reference book for those who want to know about a specific vectorborne or rodentborne disease in Europe or North America through 2003–2004. The only weakness I could find is that although the word "control" is used in the title, the book contains relatively little information on this subject. However, the depth of other areas compensates for a lack of information about control.

Overall, I recommend this to anyone who needs a reference book on vectorborne and rodentborne diseases. All we need now is an equivalent reference for such diseases in Africa, Asia, Australasia, and South America!

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Constant Troyon (1810–1865). On the Way to the Market (1859). Oil on canvas (260.5 cm × 211 cm). The State Hermitage Museum, St. Petersburg, Russia

'To Market to Market...' and Risk for Global Disease

Polyxeni Potter*

"…[T]he sound of water escaping from mill-dams… old rotten planks, slimy posts, and brick work, I love such things…. As long as I do paint, I shall never cease to paint such places. They have always been my delight," wrote John Constable (1776–1837), English landscape painter and source of inspiration for Constant Troyon and others, who looked for subjects not in the classical or academic tradition but in natural surroundings (*1*).

Bucolic farms, rivers, trees, animals, and the common people populated 19th-century French painting as fields, streams, and rural life seemed palatable alternatives to the ravages of industrial development. Artists, among them Charles-François Daubigny, Théodore Rousseau, Jules Dupré, Narciso Diaz del la Peña, rejecting the urban scene, migrated to the countryside south of Paris, near Fontainebleau Forest, to live and work in the small village of Barbizon. There, eschewing modernity and its unwelcome transformations, they sought their own style and in the process laid the foundation for realism and, later, impressionism (*2*).

The Barbizon school, as these artists became known, took the studio outdoors, in *plein air*, where the landscape ceased to be just the backdrop of classical or historical scenes and became a subject in its own right. And, recalling 17th-century Dutch traditions, it contained animals and peasant farmers engaged in everyday activities.

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Among the first Barbizon artists to become successful, Troyon became known as one of the best animal painters of his time. A native of Sèrves, he learned painting at the porcelain manufactory, where his father and grandfather were painters. Porcelain painting served him well, not for its exacting technique and judicious use of color alone but as back-up whenever he needed support to travel with other landscapists (Louis Cabat, Camille Roqueplan) and to paint the countryside around Sèrves and farming landscapes of Brittany and Normandy.

In the 1830s, Troyon started exhibiting in the Salon, where his landscapes attracted much attention among experts and the public. He was able to travel to Holland and see the masterpieces of the Dutch Golden Age. Inspired by works of Aelbert Cuyp, Esaias van de Velde, and Paulus Potter, he became more and more interested in painting animals: sheep flocks on country roads, oxen in plowing scenes. In his paintings, often large and imposing, landscapes were identified by the animals they sustained: Normandy by dairy cows grazing the lowlands, Fontainebleau by hunting dogs, recalling royal hunting parties at a nearby chateau (*3*). These tranquil landscapes, painted in a casual unaffected style, gained him popularity abroad.

Some of Troyon's later works, among them On the Way to the Market, on this month's cover, were recognized masterpieces. He was decorated with the Legion of Honor and counted Napoleon III as his patron. His last exhibit in

the Salon was in 1859. He became ill with the paralytic symptoms of venereal infection. The disease progressed to fits of madness for which he was confined to an asylum. He died soon afterwards (*4*).

The rise of landscape painting in the midst of industrial development in 19th-century France reflected general angst about the intrusion of machines and disruptions of constant change. For the Barbizon painters, the landscape was an opportunity to be in nature, probe its mysteries, "…lie on fern or withered heath," find "better worlds" (*5*).

So it was for Troyon, who viewed nature as a sanctuary, where animals and humans could live harmoniously. On the Way to the Market is a wistful image of country life at its peaceful best. Herd and keepers are traveling in the early morn. Awash in light, they appear through the mist, shadows long, breaths visible in the cold air, gait stiff, and uncertain from sleep. The woman is turned amiably toward her traveling companion. The dog jumping in the foreground is not troubling the cow nearby.

The pastoral idyll, originated in the third century by Theocritus in his accounts of the lives of Sicilian shepherds and painted so eloquently by Constable and Troyon, captured the imagination of an urban population longing for rural bliss. The perception and popularity of animals in parks and zoos, on the farm, as well as in paintings became the barometer of cultural change and the means to forge the broken bond with nature.

Late 18th- and early 19th-century poetry went down the same path. In "Home at Grasmere," William Wordsworth spoke of a "mysterious" human-animal connection, "Mysteries of passion which have made, / And shall continue evermore to make…/ One brotherhood of all the human race." And in "The Rime of the Ancient Mariner," Samuel Taylor Coleridge recounted the travails of a hero, who having capriciously killed an albatross, suffered horribly and learned that, "He prayeth best, who loveth best / All things both great and small; / For the dear God who loveth us, / He made and loveth all" (*6*).

The uneasy balance between wilderness and civilization, upset in the 19th century by the loss of virgin land to cities, is still precarious, and our relationship with nature has grown more complex. Humans, animals, and goods have reached the remotest niches, eliminating the pastoral in ways Theocritus could not have imagined. And the mysterious "brotherhood" of all creatures envisioned by Wordsworth manifests itself in ubiquitous zoonotic connections.

Recent findings suggest that animals may be susceptible to human norovirus (*7*). In an unexpected turn on the way to market, bovine imports from the United Kingdom, a major source of human exposure to bovine spongiform encephalopathy, may have contributed to global risk for this disease (*8*). And genetic resistance of sheep with ARR/ARR prion genotype to the "classic scrapie" agent is not absolute, as we thought, despite culling and massive breeding efforts (*9*). As ever connected to and vulnerable in nature, we at times damage our fellow creatures to survive. Or, as Coleridge put it, "Ours is the reptile's lot, much toil, much blame, / Manifold motions making little speed, / And to deform and kill the things whereon we feed" (*6*).

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Upcoming Issue

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Coronaviruses in North American Bats

Landscape Elements and Hantaan Virus–related Hemorrhagic Fever, China

Precautionary Behavior in Response to Perceived Threat of Pandemic Influenza

HIV, Hepatitis C, and Hepatitis B Infections in Injection Drug Users, Afghanistan

Simian Foamy Virus Transmission from Apes to Humans, Rural Cameroon

Viliuisk Encephalomyelitis in Traditional and New Geographic Regions

Spectrum of Infection and Risk Factors for Human Monkeypox, United States, 2003

Effect of Interventions on Influenza A (H9N2) Isolation in Live Poultry Markets, Hong Kong

Detecting Human-to-Human Transmission of Influenza A (H5N1)

Influenza (H5N1) with Decreased Oseltamivir Sensitivity, Indonesia

Molecular Typing and Cutaneous Leishmaniasis, Morocco

Mokola Virus in Domestic Mammals, South Africa

Tuberculosis in Children and Adolescents, Taiwan, 1996–2003

Complete list of articles in the September issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

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Chicago, IL, USA
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JOURNAL BACKGROUND AND GOALS

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Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
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The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

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?

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   - 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.

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   - 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.
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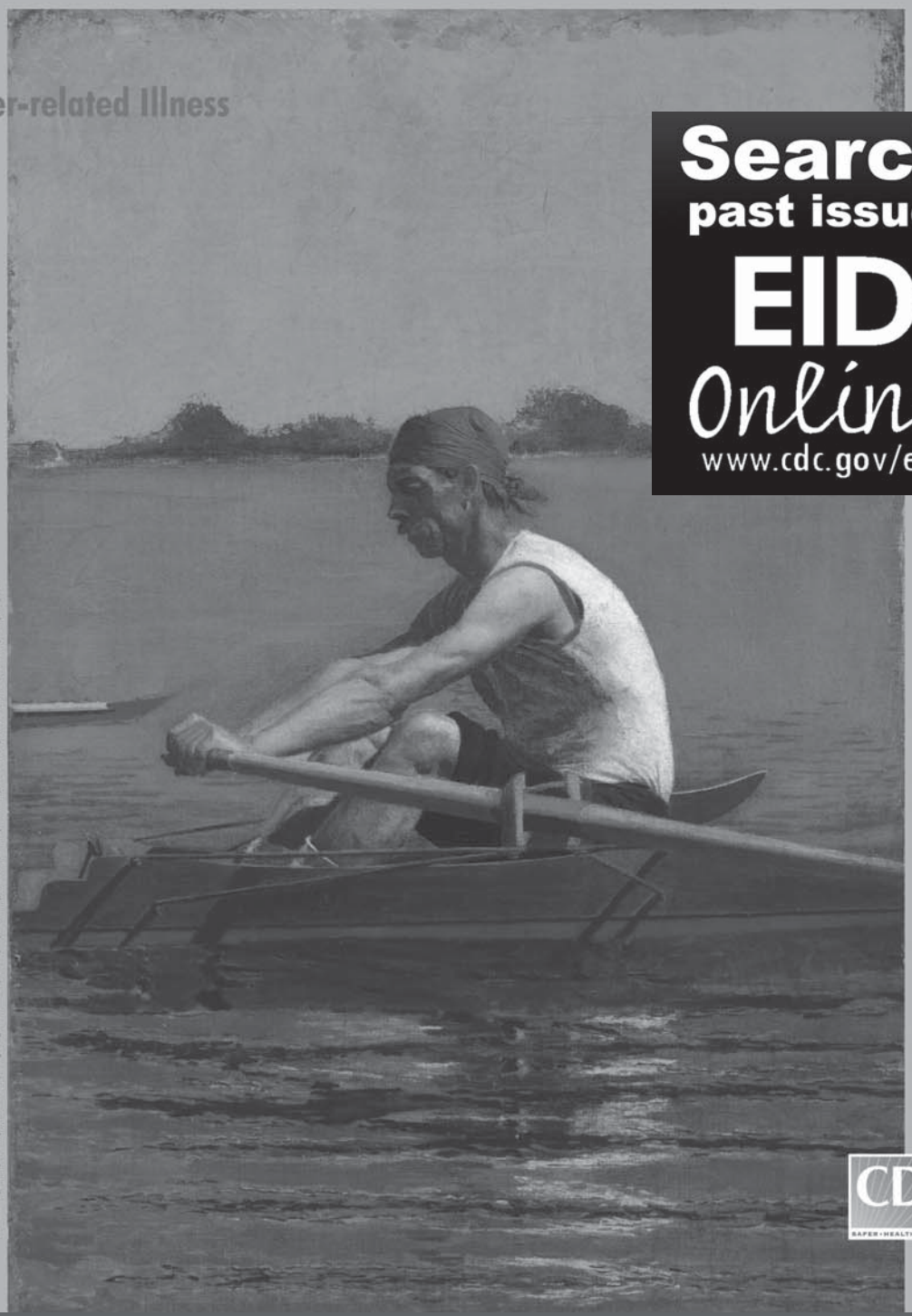

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Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online 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.