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EMERGING INFECTIOUS DISEASES

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The Garden of Eden (1618)
Oil on wood (55 cm × 107 cm)
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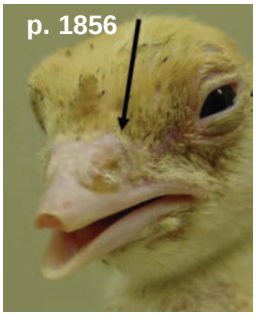

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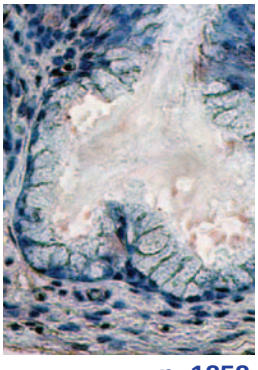

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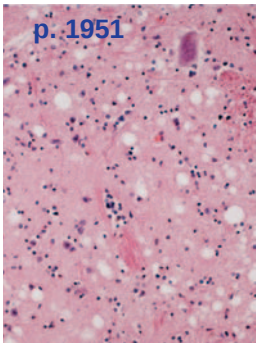
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International Attention for Zoonotic Infections

Nina Marano,* Paul Arguin,* Marguerite Pappaioanou,† Bruno Chomel,‡ Esther Schelling,§ Vincent Martin,¶ Jay C. Butler,* C. Ben Beard,* and Lonnie King*

Snakes on a Plane was poised to be the blockbuster movie of the 2006 summer season. It featured reptiles smuggled onto an airplane as an unusual bioweapon of mass destruction. In a much less dramatic fashion, this absurd scenario plays out every day, when tons of live animals and unprocessed animal products are shipped internationally around the globe, providing many opportunities for rapidly translocating zoonotic pathogens. Episodes of emerging zoonoses are being increasingly recognized around the world. From 1996 to 2004, some 21% of 10,490 reports of animal diseases from 191 countries submitted to the Program for Monitoring Emerging Diseases (ProMED) concerned humans affected by zoonotic disease (*1*). This zoonoses theme issue of Emerging Infectious Diseases (EID) corroborates this finding, presenting reports of zoonotic disease from all corners of the globe, including the People's Republic of China, Vietnam, Slovakia, Indonesia, the United States, Israel, Bangladesh, the Netherlands, Brazil, Algeria, India, the Democratic Republic of the Congo, and Italy.

As public health and animal health organizations attempt to respond to these emerging and reemerging zoonotic diseases, their ability and skill in forming new strategic partnerships are of paramount importance. This year was highlighted by the establishment of the Centers for Disease Control and Prevention (CDC) as a World Organization for Animal Health (OIE) Collaborating Center for Emerging and Remerging Zoonoses.

To initiate this collaborating center, the International Symposium on Emerging Zoonoses (ISEZ) was held in conjunction with the International Conference on Emerging Infectious Diseases and the International Conference on Women and Infectious Diseases in Atlanta in March 2006. ISEZ was cosponsored by OIE, the United Nations Food and Agriculture Organization, the US Department of Agriculture Animal and Plant Health Inspection Service, the Department of Interior US Geological Survey, National Wildlife Health Center, the World Health Organization, and CDC. ISEZ was attended

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Guest Editors

Nina Marano

Dr Marano is the chief of the Geographic Medicine and Health Promotion Branch in the Division of Global Migration and Quarantine at CDC. The branch mission is to protect the health of US travelers at home and abroad and to prevent the introduction of zoonotic diseases into the country through imported animals and animal products.

Paul Arguin

Dr Arguin is the chief of the Domestic Response Unit in the Malaria Branch within the National Center for Zoonotic, Vector-borne, and Enteric Diseases at CDC. His research interests include the prevention and treatment of infectious diseases associated with international travel, including malaria and zoonoses.

Marguerite Pappaioanou

Dr Pappaioanou is professor of infectious disease epidemiology in the School of Public Health with a joint appointment in the College of Veterinary Medicine at the University of Minnesota. Her areas of interest are in emerging zoonotic infectious diseases, with a special interest in influenza viruses and in collaborative efforts that bridge public health and domestic animal and wildlife health sectors that address emerging zoonotic infectious diseases.

Jay C. Butler

Dr Butler is on detail from CDC to the Alaska Division of Public Health, where he is deputy director for science and medicine and is the state epidemiologist. His research interests include vaccine-preventable diseases, antimicrobial drug resistance, emerging infectious diseases, microbial ecology, and emergency preparedness planning and response.

by >400 veterinarians, physicians, and public health professionals from all over the world. The objective of the symposium was to share information among public health and animal health professionals so that more effective and cooperative partnerships could be developed. In turn, this would help to better understand, prevent, and control new microbial dangers to human and animal health that occur globally every day.

The 44 internationally renowned ISEZ speakers and moderators recognized the spirit in which this meeting was created. Their participation reflected a true commitment to the partnership between public health and animal health needed to meet the ever-growing microbial challenges threatening human, animal, and environmental health on a seemingly continual basis. The speakers lent their talent and expertise to the following themes: the epidemiology of pathogens and diseases shared among humans and animals, the risks of wildlife and exotic pet trade to human and animal health, the effects of agricultural practices on human and ecosystem health, the lessons learned from previous experiences, and collaborative achievements. Speakers offered solutions that had been tested through innovative, interdisciplinary, and intersectoral research to deal with the problems of emerging zoonoses. A few presentations are highlighted below.

In his presentation, Emerging and Remerging Zoonoses from Wildlife Reservoirs to Exotic Pets, Bruno Chomel, University of California, Davis, College of Veterinary Medicine, reminded participants that most emerging infectious diseases are zoonotic, with wildlife constituting a large and often unknown reservoir. Wildlife can also be a source for reemergence of previously eradicated zoonoses. The discovery of such zoonoses is often related to better diagnostic tools. However, human modification to natural wildlife habitats and human behavior, such as deforestation, which causes range expansion of the tick vector for Kyasanur Forest disease in India, also create opportunities for emergence of zoonotic diseases. Translocation of raccoon dogs from Asia into Europe has provided a new potential rabies reservoir. Human behavior includes sleeping with pets, reported by 30% of Americans, and keeping of exotic pets that provide opportunities for microbial transmission that have not existed until recently. More than 10,000 pet tigers are kept in the United States—more tigers than are currently living in the wild throughout the world. Thus, addressing human influences on ecosystems and behavior with regard to wild and exotic animals must be incorporated into efforts at preventing and controlling emerging diseases that involve wildlife and other valued natural resources.

In her presentation, Combined Vaccination Delivery to Remote and Mobile Pastoral Families and Their Animals, Esther Schelling, Swiss Tropical Institute, focused on the potential of joining public health and veterinary services to achieve higher vaccination coverage in Africa's remote rural settings. Such sharing is aimed at reducing vaccine-preventable diseases in persons and their livestock. Her findings from Chad indicated that sharing transport logistics and cold chain equipment between the public health and veterinary sectors in the most remote areas reduced total program delivery costs, was highly valued by pastoralists, and was an important strategy to connect hard-to-reach populations with needed vaccinations such as for measles and polio for children and anthrax for livestock. By optimizing the use of limited logistic and human resources, public health and veterinary services were strengthened, especially at the district level, and, in turn, the services can become more prepared to respond to endemic and epidemic diseases.

ISEZ participants learned about the impact of human behavior on emergence and subsequent spread of highly pathogenic avian influenza (HPAI) H5N1 strain in Asia from Vincent Martin of the Food and Agriculture Organization. Human behavior that supported disease emergence includes agriculture practices that allow species mixing, limited biosecurity to prevent mixing of domestic and wild animals, and a cultural preference for warm meat that has made live bird markets common in urban areas. These factors act synergistically with the intrinsic characteristics of the virus and the rapid evolution of animal and farming production systems in the region to support emergence. Martin emphasized that understanding the underlying farming practices and cultural preferences that influenced the emergence and spread of HPAI in developing countries has been instrumental in implementing effective risk reduction measures. The experience of fighting HPAI in Asia shows that control efforts should be focused on production sectors with low biosecurity standards and free-ranging chickens and ducks. Creating conditions that are not conducive to microbial selection and emergence represents a major challenge, from a disease management and cultural point of view, for reducing the risk for avian influenza occurrence and subsequent human infection.

This issue of EID continues in the tradition of the 2 previous December theme issues by focusing on zoonoses, reminding readers again that we must maintain a vigilance in combatting these microbial threats. The issue highlights efforts directed at identifying disease reservoirs and finding better ways to understand, evaluate, prevent, and control disease transmission. At the same time, healthy ecosystems need to be promoted, economic livelihoods safeguarded, and cultural beliefs respected at household to national and international levels. Contained in this issue are articles based on the following ISEZ presentations: Review of Bats and SARS (*2*) and Ecologic Niche Modeling and Spatial Patterns of Disease Transmission

(*3*). Also featured are articles that focus on the role of risk factors for introducing zoonotic diseases, such as monkeypox associated with domestic trade in certain animal species and human behavior as a risk factor for exposure to avian influenza in Vietnam. Many species of animals are highlighted, including cats colonized with methicillin-resistant *Staphylococcus aureus*, horses and wild mammals infected with West Nile virus, dogs with rickettsial infections, turkeys with human metapneumovirus, and nonhuman primates infected with malaria. We encourage our readers to continue to conduct and submit the findings of essential research on emerging zoonotic diseases to EID as we strive to share and disseminate this information to our multidisciplinary readership.

References

1. Cowen P, Garland T, Hugh-Jones ME, Shimshony A, Handysides S, Kaye D, et al. Evaluation of ProMED-mail as an electronic early warning system for emerging animal diseases: 1996 to 2004. J Am Vet Med Assoc. 2006;229:1090–9.
2. Wang L-F, Shi Z, Zhang S, Field H, Daszak P, Eaton BT. Review of bats and SARS. Emerg Infect Dis. 2006:12:1834–40.
3. Peterson AT. Ecologic niche modeling and spatial patterns of disease transmission. Emerg Infect Dis. 2006;12:1822–6.

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On the Question of Sporadic or Atypical Bovine Spongiform Encephalopathy and Creutzfeldt-Jakob Disease

Paul Brown,* Lisa M. McShane,† Gianluigi Zanusso,‡ and Linda Detwiler§

Strategies to investigate the possible existence of sporadic bovine spongiform encephalopathy (BSE) require systematic testing programs to identify cases in countries considered to have little or no risk for orally acquired disease or to detect a stable occurrence of atypical cases in countries in which orally acquired disease is disappearing. To achieve 95% statistical confidence that the prevalence of sporadic BSE is no greater than 1 per million (i.e., the annual incidence of sporadic Creutzfeldt-Jakob disease [CJD] in humans) would require negative tests in 3 million randomly selected older cattle. A link between BSE and sporadic CJD has been suggested on the basis of laboratory studies but is unsupported by epidemiologic observation. Such a link might yet be established by the discovery of a specific molecular marker or of particular combinations of trends over time of typical and atypical BSE and various subtypes of sporadic CJD, as their numbers are influenced by a continuation of current public health measures that exclude high-risk bovine tissues from the animal and human food chains.

Bovine spongiform encephalopathy (BSE) was first recognized in 1986 in the United Kingdom and quickly reached epidemic proportions, affecting >30,000 cattle per year by 1992. Because of continuing exportation of both live cattle and meat and bone meal rendered from the carcasses of slaughtered cattle, the disease spread throughout most of Europe and a few non-European countries. By 2006, 20 years after its first appearance in the United Kingdom, the disease had been reported in an additional 24 countries (*1*).

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Beginning toward the end of the 1980s in the United Kingdom, and in the 1990s in other countries, numerous regulations were enacted to minimize the entry of contaminated tissues into both the animal and human food chains and to eliminate the international spread of disease. These measures have been extraordinarily successful, to the extent that no new countries have been added to the list during the past year and the number of new cases has dramatically diminished in most countries in which BSE has appeared (the situation in some countries with insufficient surveillance remains unclear).

Although the origin of the epidemic is thought to have been caused by a species-crossing contamination by sheep scrapie during the course of rendering and recycling carcass meat and bone meal as cattle feed, an alternative hypothesis suggested an origin in a similarly recycled case of spontaneously occurring disease in cattle. The pros and cons of these competing ideas have been argued elsewhere (*2*,*3*), and neither will ever be convincingly proved or disproved. Thus, the phenomenon of spontaneous disease remained in limbo until the recent discovery of "atypical" strains of BSE reopened the question. In this article we consider the importance of atypical BSE within the overall concept of sporadic (spontaneous) disease and whether such cases, if they exist, could account for at least some cases of apparently sporadic Creutzfeldt-Jakob (CJD) in humans.

Sporadic BSE

Obviously, the ideal country in which to examine the question of sporadic BSE would have a large national herd that was guaranteed never to have been exposed to environmental sources of infection. Such an ideal will never be realized. Until recently, the United States appeared to have at least approached the ideal by having a large national

herd, an adequate testing program, and an apparently small risk for contamination by imported cattle or cattle feed. That position was made vulnerable in late 2003 by the discovery of a case of BSE imported from Canada and was eliminated altogether by the subsequent discovery of 2 indigenously infected animals in widely separate regions of the country. Although the 2 indigenous cases might represent sporadic disease, the continuing identification of cases in western Canada, coupled with a history of substantial numbers of cattle imported from Canada into the United States (both indigenous US animals had the same molecular "signature" as the most recent Canadian case), makes it difficult to ignore the possibility of undetected instances of feed contamination from imported cattle and recycled infectious carcasses.

At present, the 2 best countries in which to undertake testing programs would be Argentina and Australia; both have large national herds (≈50 million and 30 million animals, respectively), and both are considered to be free of orally acquired BSE infections, on the basis of importation history, nutritional practices, and adequacy of surveillance (*4*). Even in these countries, however, the discovery of a case of BSE could not be guaranteed to be spontaneous because of the widespread global distribution of potentially infected cattle and cattle feed and the vagaries of international trade: imperfect record keeping, lack of compliance, and just plain deception.

By way of illustration, an incident occurred many years ago that involved a particularly bulky shipment labeled as a pesticide. The large quantity seemed unusual to the customs inspector, who opened it and discovered that the shipment contained meat and bone meal destined to be spread on fields to inhibit grazing by deer, a serious agricultural pest. Thus, a study of sporadic BSE would only be truly convincing if no cases were identified.

Moreover, the criteria for answering the question of sporadic BSE are different than for orally acquired BSE. Most importantly, we do not know at what age sporadic cases of BSE might occur, but they are unlikely to be in the 3- to 5-year-old age group in which orally acquired BSE is most prevalent. If the age distribution of sporadic disease in cattle were to mimic that of sporadic CJD in humans, it would not peak until 14–20 years of age (the last third of the ≈20-year natural life span of a cow). Substantial numbers of such older cattle do not exist, and thus it may never be possible to state with assurance that spontaneous BSE does not occur.

Even if we accept this practical constraint, we can still take advantage of the fact that in many countries a proportion of the total slaughter population consists of breeding stock and dairy cows that are culled at ≥7 years of age, and animals that go directly to rendering plants or die "on farm" further increase this number. Argentina, for example, with a national herd of ≈50 million cattle, in 2005 recorded nearly 1.4 million deaths from slaughter and natural causes in animals ≥7 years (L. Mascitelli, pers. comm.).

Approximately 10% of cases of sporadic CJD occur in patients 25–50 years of age (Figure 1); this age in humans corresponds to the middle third of a cow's normal life span, or 7–13 years of age. If the age distribution of sporadic BSE followed the same pattern, negative test results in a total of ≈3 million animals randomly selected from this group would allow us to be 95% confident that sporadic BSE is not present at a prevalence >1 per million, and ≈4.5 million negative animals would raise the level of confidence to 99%. Larger numbers of BSE-negative animals would be required to achieve these levels of confidence for a maximum prevalence ≤1 per 10 million cattle (Table 1, Figure 2).

Even the least rigorous negative result—a prevalence not greater than that of sporadic CJD in humans, or 1 per million—would require several years to achieve, and it is perhaps unrealistic to suppose that the motivation to prolong the testing program will endure much beyond the global disappearance of orally acquired BSE and variant CJD. Nevertheless, to the degree that testing older as well as younger adult animals approached these numbers, both statistical and consumer confidence would increase, and at the very least provide reassurance that the occurrence of sporadic disease must be exceedingly rare, with little likelihood of posing a risk to either human or animal nutrition.

Atypical BSE

Because of its contemporary nature, the study of atypical BSE is very much a work in progress, with comparatively little published data and many unknowns. The first 2

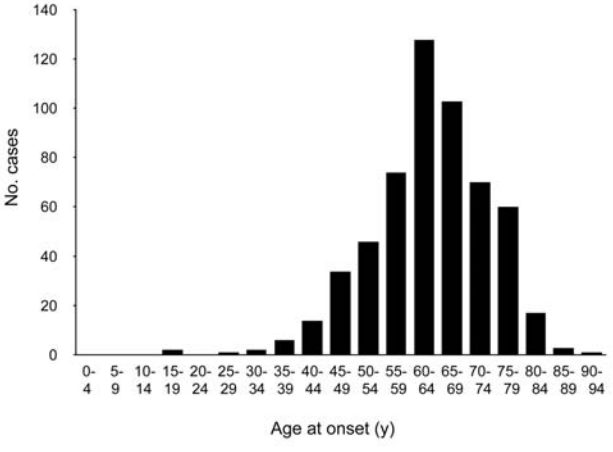

Figure 1. Distribution of ages at onset of illness in 500 cases of neuropathologically verified or experimentally transmitted sporadic Creutzfeldt-Jakob disease. Approximately 10% of cases occur in patients during the middle third (25–49 years) of a human lifespan, which corresponds to age in cattle of ≈7–13 years.

Table 1. Total number of older cattle with negative test results required to achieve 95% or 99% confidence* that sporadic cases of BSE are not present at a level higher than the illustrated prevalence rates†

Maximum prevalence	Log_{10} prevalence	No. tested cattle	
		95% Confidence	99% Confidence
1 per million	–6.000	2,995,731	4,605,168
1 per 2 million	–6.301	5,991,463	9,210,338
1 per 3 million	–6.477	8,987,195	13,815,508
1 per 4 million	–6.602	11,982,928	18,420,678
1 per 5 million	–6.699	14,978,660	23,025,849
1 per 6 million	–6.778	17,974,392	27,631,019
1 per 7 million	–6.845	20,970,124	32,236,189
1 per 8 million	–6.903	23,965,857	36,841,359
1 per 9 million	–6.954	26,961,589	41,446,529
1 per 10 million	–7.000	29,957,321	46,051,700

*α = 0.05 or 0.01.
†The required number of tests, all of which must be negative, is given by log (α)/log (1-prevalence); BSE, bovine spongiform encephalopathy.

cases to be identified were a serendipitous discovery made in the course of an unrelated experimental study that required a detailed neuropathologic and immunochemical examination of the entire brain (*5*). The absence of clinical signs in these older animals, the unusual distribution of PrP^{TSE}, together with amyloid plaques, and a Western blot pattern that differed from the stereotypic pattern seen in typical BSE left little doubt about the probability that a new "atypical strain" had been identified (bovine amyloidotic spongiform encephalopathy[BASE]).

Although no further cases were found in over 100 cattle examined in Italy, the initiation of Western blot studies of animals in other countries with screening test programs began to yield additional atypical patterns (Table 2, Figure 3) (*6–14;* P. Lind, pers. comm.). Two major patterns have been described, named L (resembling the original Italian case pattern with a lower molecular weight than typical BSE) and H (for a distinct pattern first seen in France with a higher molecular weight than typical BSE). It is not yet clear whether other mixed patterns result from technical procedures in different laboratories or whether a more complicated scheme of classification will evolve as more atypical patterns are discovered.

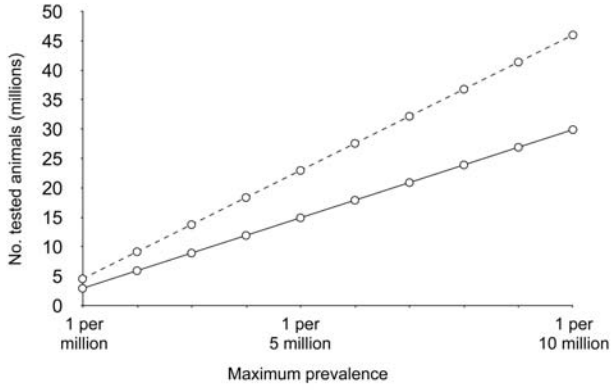

Figure 2. Maximum prevalence according to number of negative cattle at 95% (solid line) and 99% (dashed line) confidence levels. See Table 1 for exact numbers and statistical method.

In addition, Western blots of PrP^{TSE} are a fragile basis on which to define a BSE phenotype. Little or no information is available about either the clinical status or neuropathologic features of these animals. We know that cases have occurred in different breeds and PrP genotypes, and we also know that very few of the animals have had the typical clinical picture of BSE (behavioral disturbances, sensory signs, ataxia, and tremors), but a cloud of ambiguity surrounds the clinical picture even in those animals for which an extensive post-hoc investigation was undertaken. The fact that few detailed neuropathologic results are available is explained by the need to preserve at least a full half brain for examination, which is presently not done in any of the various countries that have screening test programs. In the future, the brain as well as the carcass must be retained in cold storage until the test results are known.

The frequency of atypical cases is another unknown. Published (*7,12*) and unpublished (*11,13*) observations indicate that in some countries it might be as high as 5%–10% of the total number of older animals diagnosed by rapid screening tests (e.g., 2/27 in Germany, and 1/9 in Canada), which would seem to be a surprisingly high proportion of spontaneously occurring cases. However, data are not yet sufficient to estimate the overall prevalence of atypical BSE, i.e., cases per million tested animals of all ages.

In this context, a word is in order about the US testing program. After the discovery of the first (imported) cow in 2003, the magnitude of testing was much increased, reaching a level of >400,000 tests in 2005 (Figure 4). Neither of the 2 more recently indigenously infected older animals with nonspecific clinical features would have been detected without such testing, and neither would have been identified as atypical without confirmatory Western blots. Despite these facts, surveillance has now been decimated to 40,000 annual tests (USDA news release no. 0255.06, July 20, 2006) and invites the accusation that the United States will never know the true status of its involvement with BSE.

Table 2. Summary of atypical cases of bovine spongiform encephalopathy (BSE)

Country	Age, y	Breed	Symptoms	Neuropathology		Western blot pattern*
				Spongiform changes	Immunohistochemistry	
Italy	11	Bruna Alpina	None	Mild	Plaques	L
	15	Piemontese	None	Mild	Plaques	L
Denmark	14	Charolais	None	NR	NR	L
Poland	12	Black-white breed	None	Present	Positive (no plaques)	L
Japan	2	Holstein	None	Absent	Negative	L_1
	14	Japanese Black	Dystasia	Severe	Positive (no plaques)	H
Belgium	5.5	East-Flemish	None	Absent	Negative	L_1
France	10	Cross breed	None	NR	NR	H
	15	Prim Holstein	None	NR	NR	H
	8	Charolais	None	NR	NR	H
The Netherlands	13	Black-white Holstein, Freisian	NR	Present	No plaques	H
Sweden	12	Mixed Charolais	Recumbent	NR	Positive (no plaques)	H_1
Switzerland	19	Zebu	Typical BSE	Typical BSE	Positive (no plaques)	H
Germany	13	Angus	NR	Absent	Positive (no plaques)	H
	15	Holstein-Freisian	NR	Absent	Positive (no plaques)	L
USA	12	Brahma cross	Falling	Absent	No plaques	H
	10	Red crossbred	Recumbent	Absent	No plaques	H
Canada	16	Charolais	Recumbent	NR	Positive (no plaques)	H

*L, lower molecular weight; H, higher molecular weight (the 2 major Western blot PrP glycopatterns that distinguish the strains from each other and from the pattern seen in typical BSE); NR, not reported. Only the Italian cows and Swiss zebu had full neuropathologic examinations (others were limited to examination of the obex). Details are not available for additional animals with both H and L strains in France and Poland.

In short, a great deal of further work will need to be done before the phenotypic features and prevalence of atypical BSE are understood. More than a single strain may have been present from the beginning of the epidemic, but this possibility has been overlooked by virtue of the absence of widespread Western blot confirmatory testing of positive screening test results; or these new phenotypes may be found, at least in part, to result from infections at an older age by a typical BSE agent, rather than neonatal infections with new "strains" of BSE. Neither alternative has yet been investigated.

Sporadic CJD

The possibility that at least some cases of apparently sporadic CJD might be due to infection by sporadic cases of BSE cannot be dismissed outright. Screening programs needed to identify sporadic BSE have yet to be implemented, and we know from already extant testing programs that at least a proportion of infected animals have no symptoms and thus would never be identified in the absence of systematic testing. Thus, sporadic BSE (or for that matter, sporadic disease in any mammalian species) might be occurring on a regular basis at perhaps the same annual frequency as sporadic CJD in humans, that is, in the range of 1 case per million animals.

Whether humans might be more susceptible to atypical forms of BSE cannot be answered at this time. Experimentally transmitted BASE shows shorter incubation periods than BSE in at least 1 breed of cattle, bovinized transgenic mice, and *Cynomolgus* monkeys (*12*,*13*). In humanized transgenic mice, BASE transmitted, whereas typical BSE did not transmit (*13*). Paradoxically, the other major phenotype (H) showed an unusually long incubation period in bovinized transgenic mice (*12*).

The limited experimental evidence bearing on a possible relationship between BSE and sporadic CJD is difficult to interpret. The original atypical BASE strain of BSE had a molecular protein signature very similar to that of 1 subtype (type 2 M/V) of sporadic CJD in humans (*5*). In another study, a strain of typical BSE injected into humanized mice encoding valine at codon 129 showed a glycopattern indistinguishable from the same subtype of sporadic CJD (*15*). In a third study, the glycopatterns of

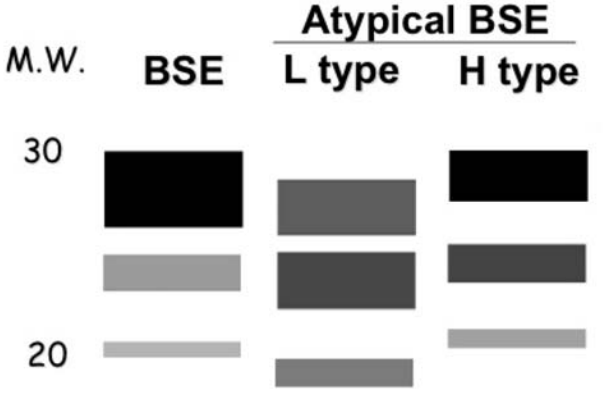

Figure 3. Representation of Western blots of PrP^{TSE} patterns of typical bovine spongiform encephalopathy (BSE) and the 2 major types of atypical BSE. M.W., molecular weight in kilodaltons; L type, atypical "light" pattern; H type, atypical "heavy" pattern.

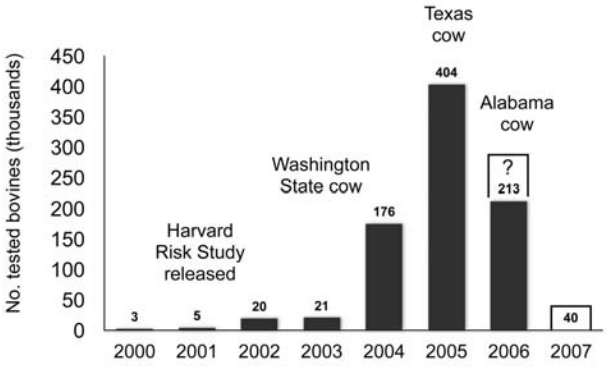

Figure 4. Numbers of tested cattle in the United States, 2000–2007. Number tested in 2006 as of August 20; number tested in 2007 proposed by the US Department of Agriculture.

both the H and L strains of atypical BSE evidently did not resemble any of the known sporadic CJD subtypes (*12*).

To these molecular biology observations can be added the epidemiologic data accumulated during the past 30 years. The hypothesis that at least some cases of apparently sporadic CJD are due to unrecognized BSE infections cannot be formally refuted, but if correct, we might expect by now to have some epidemiologic evidence linking BSE to at least 1 cluster of apparently sporadic cases of CJD. Although only a few clusters have been found (and still fewer published), every proposed cluster that has been investigated has failed to show any common exposure to bovines. For that matter, no common exposure has been shown to any environmental vehicles of infection, including the consumption of foodstuffs from bovine, ovine, and porcine sources, the 3 livestock species known to be susceptible to transmissible spongiform encephalopathies. Additional negative evidence comes from several large case-control studies in which no statistically significant dietary differences were observed between patients with sporadic CJD and controls (*16*,*17*).

On the other hand, the difficulty of establishing a link between BSE and CJD may be compounded by our ignorance of the infectious parameters of a sporadic form of BSE (e.g., host range, tissue distribution of infectivity, route of transmission, minimum infectious dose for humans, whether single or multiple). Presumably, these parameters would resemble those of variant CJD; that is, high infectivity central nervous system and lymphoreticular tissues of an infected cow find their way into products consumed by humans. Transmissions that might have occurred in the past would be difficult to detect because meat products are generally not distributed in a way that results in detectable geographic clusters.

Barring the discovery of a specific molecular signature (as in variant CJD), the most convincing clue to an association will come from the observation of trends over time of the incidence of typical and atypical BSE and of sporadic and variant CJD. With 4 diseases, each of which could have increasing, unchanging, or decreasing trends, there could be 81 (3^4) possible different combinations. However, it is highly likely that the trends for typical BSE and variant CJD will both decrease in parallel as feed bans continue to interrupt recycled contamination. The remaining combinations are thus reduced to 9 (3^2), and some of them could be highly informative.

For example, if the incidence of atypical BSE declines in parallel with that of typical BSE, its candidacy as a sporadic form of disease would be eliminated (because sporadic disease would not be influenced by current measures to prevent oral infection). If, on the other hand, atypical BSE continues to occur as typical BSE disappears, this would be a strong indication that it is indeed sporadic, and if in addition at least 1 form of what is presently considered as sporadic CJD (such as the type 2 M/V subtype shown to have a Western blot signature like BASE) were to increase, this would suggest (although not prove) a causal relationship (Figure 5).

Recognition of the different forms of BSE and CJD depends upon continuing systematic testing for both bovines and humans, but bovine testing will be vulnerable to heavy pressure from industry to dismantle the program as the commercial impact of declining BSE cases ceases to

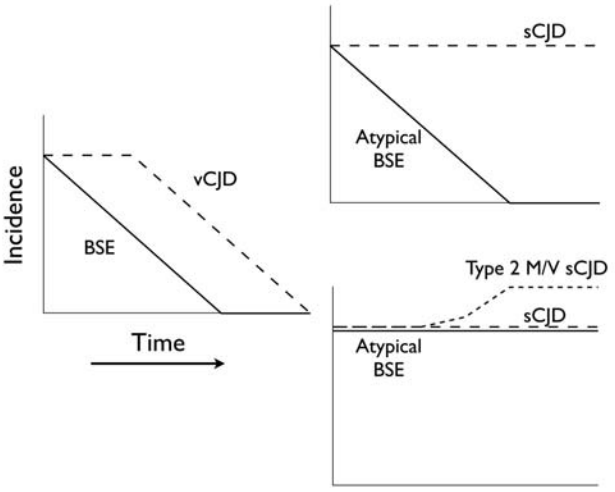

Figure 5. Diagram of 2 possible informative trends in the incidence of bovine spongiform encephalopathy (BSE) and Creutzfeld-Jakob disease (CJD). The left panel shows the likely trends of typical BSE and variant CJD (vCJD). The right upper panel shows 1 possible pair of trends of atypical BSE and sporadic CJD (sCJD) that might occur in conjunction with the typical BSE/vCJD trends, and would be consistent with the interpretation that atypical BSE is not sporadic and is not related to sCJD. The right lower panel shows a second possible associated pair of trends consistent with the interpretation that atypical BSE is sporadic and might also be related to the type 2 M/V subset of apparently sCJD.

be an issue. Industry should be aware, however, of the implications of sporadic BSE. Its occurrence would necessitate the indefinite retention of all of the public health measures that exclude high-risk bovine tissues from the animal and human food chains, whereas its nonoccurrence would permit tissues that are now destroyed to be used as before, once orally acquired BSE has disappeared.

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Dr Brown recently retired from the Laboratory of CNS Studies at the National Institutes of Health

References

1. World Organization for Animal Health. Bovine spongiform encephalopathy. Geographical distribution of countries that reported BSE confirmed cases since 1989 [cited 2006 Oct 24]. Available from http://www.oie.int/eng/info/en_esb.htm
2. Brown P, Bradley R, Detwiler L, Dormont D, Hunter N, Wells GAH, et al. Transmissible spongiform encephalopathy as a zoonotic disease. International Life Sciences Institute (ILSI) Europe Report Series. Brussels: ILSI Press; 2003.
3. Horn GM, Bobrow ME, Bruce M, Goedert M, McLean A, Webster J. Review of the origin of BSE 2001, London: Stationery Office; 2001.
4. World Organization for Animal Health. Bovine spongiform encephalopathy. Recognition of the bovine spongiform encephalopathy status of member countries [cited 2006 Oct 24]. Available from http://www.oie.int/eng/info/en_statesb.htm
5. Casalone C, Zanusso G, Acutis P, Ferrari S, Capucci L, Tagliavini F, et al. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. Proc Natl Acad Sci U S A. 2004;101:3065–70.
6. Danish Institute for Food and Veterinary Research [cited 2006 Oct 24]. Available from http://www.dfvf.dk/Default.asp?ID=8147&M=News&PID=89507&NewsID=792
7. Polak M, Rozek W, Rola J, Zmudzinski JF. Prion protein glycoforms from BSE cases in Poland. Bulletin of the Veterinary Institute of Pulawy. 2004;48:201–5.
8. De Bosschere H, Roels S, Vanopdenbosch E. Atypical case of bovine spongiform encephalopathy in an East-Flemish cow in Belgium [cited 2006 Oct 24]. Int J Appl Res Vet Med. 2004;2:52–4. Available from http://www.jarvm. com/articles/Vol2Iss1/DEBOSSCHERE.htm
9. Yamakawa Y, Hagiwara K, Nohtomi K, Nakamurua Y, Nishijima M, Higuchi Y, et al. Atypical proteinase K–resistant prion protein (PrPres) observed in an apparently healthy 23-month-old Holstein steer. Jpn J Infect Dis. 2003;56:221–2.
10. Biacabe AG, Laplanche JL, Ryder S, Baron T. Distinct molecular phenotypes in bovine prion diseases. EMBO Rep. 2004;5:110–4.
11. Canadian Food Inspection Agency. Report on the investigation of the sixth case of bovine spongiformencephalopathy (BSE) in Canada [cited 2006 Oct]. Available from http://www.inspection.gc.ca/english/anima/heasan/disemala/bseesb/mb2006/6investe.shtml
12. Buschmann A, Gretzshel A, Biacabe AG, Schiebel K, Corona C, Hoffmann C, et al. Atypical BSE in Germany—proof of transmissibility and biochemical characterization. Vet Microbiol. 2006; 117:103–16.
13. Book of abstracts. Prion 2006, International Conference on Prion Diseases of NeuroPrion, Network of Excellence, Turin, Italy, 2006 Oct 3–6 [cited 2006 Oct 24]. Available from http://www.neuroprion.com/en/ev_prion 2006.html
14. Seuberlich T, Botteron C, Wenker C, Café-Marçal V, Oevermann A, Haase B, et al. Spongiform encephalopathy in a miniature zebu. Emerg Infect Dis. 2006;12::1950–30.
15. Wadsworth JDF, Asante EA, Desbruslais M, Linehan JM, Joiner S, Gowland I, et al. Human prion protein with valine 129 prevents expression of variant CJD phenotype. Science. 2004;306:1793–6.
16. Wientjens DP, Davanipour Z, Hofman A, Kondo K, Matthews WB, Will RG, et al. Risk factors for Creutzfeldt-Jakob disease: a reanalysis of case control studies. Neurology. 1996;46:1267–91.
17. Van Duijn CM, Delasnerie-Lauprêtre N, Masullo C, Zerr I, de Silva R, Wientjens DPWM, et al. Case-control study of risk factors of Creutzfeldt-Jakob disease in Europe during 1993–1995. Lancet. 1998;351:1081–5.

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Ecologic Niche Modeling and Spatial Patterns of Disease Transmission

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Ecologic niche modeling (ENM) is a growing field with many potential applications to questions regarding the geography and ecology of disease transmission. Specifically, ENM has the potential to inform investigations concerned with the geography, or potential geography, of vectors, hosts, pathogens, or human cases, and it can achieve fine spatial resolution without the loss of information inherent in many other techniques. Potential applications and current frontiers and challenges are reviewed.

The emerging and evolving field of landscape epidemiology has explored techniques for summarizing spatial patterns in disease transmission data. These techniques seek spatial patterns at some level of generalization or averaging and then summarize overall patterns and trends in the form of a smoothed surface. Techniques typically applied to these challenges include splining and kriging, as well as smoothing based on average values within coarser-grained windows across landscapes (*1*–*3*). These approaches always involve some loss of resolution to smooth the surfaces, and some degree of averaging is involved (Figure).

Although these approaches provide simple summaries of spatial patterns, they do not often succeed in illustrating true levels of complexity and heterogeneity that characterize biologic landscapes. Disease transmission cycles are composite phenomena that represent interactions between sets of species: hosts, vectors, and pathogens. The complexities of spatial occurrence of disease will represent the combination of complexities of occurrence of the component species, as well as effects of chance events. Thus, broad-trend generalizations such as those produced using the smoothing techniques mentioned above are unlikely to lead to novel insights and new understanding of complex systems. The approach advocated in this report improves the pattern summary by estimating species-specific ecologic niches. In this way, the complex influences of environmental variation on species' distributions and their translation into disease transmission patterns can be appreciated in greater detail (Figure).

Ecologic Niche Modeling (ENM)

Joseph Grinnell originated the concept of ecologic niches and was the first to explore the connections between ecologic niches and geographic distributions of species (*4*). His idea, translated into more modern terminology, was that the ecologic niche of a species is the set of conditions under which the species can maintain populations without immigration of individuals from other areas. A more complete discussion of the concept of ecologic niches and their mapping onto the geographic distributions of species has been provided elsewhere (*5*).

Use of the ENM approach has grown considerably in the biodiversity community in recent years (*6*–*10*). The idea is that known occurrences of species across landscapes can be related to raster geographic information system coverages summarizing environmental variation across those landscapes to develop a quantitative picture of the ecologic distribution of the species. ENM characterizes the distribution of the species in a space defined by environmental parameters, which are precisely those that govern the species' geographic distribution under Grinnell's definition.

A particular strength of ENM is its independence from any particular landscape. ENM can be used to identify potential distributional areas on any landscape: unsampled or unstudied portions of the native landscape, areas of actual or potential invasion by a species with an expanding range, or changing potential distributional areas as a consequence of change (e.g., land use change or climate

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change). Thus, ENM represents a powerful tool for characterizing ecologic and geographic distributions of species across real-world landscapes.

Applications to Disease Systems

In recent years, the ENM approach has seen several prototype applications to disease transmission systems by public health and epidemiology specialists who have been willing to explore novel ideas and approaches. I outline what the technique has to offer to the field and provide citations of example publications for each benefit and use.

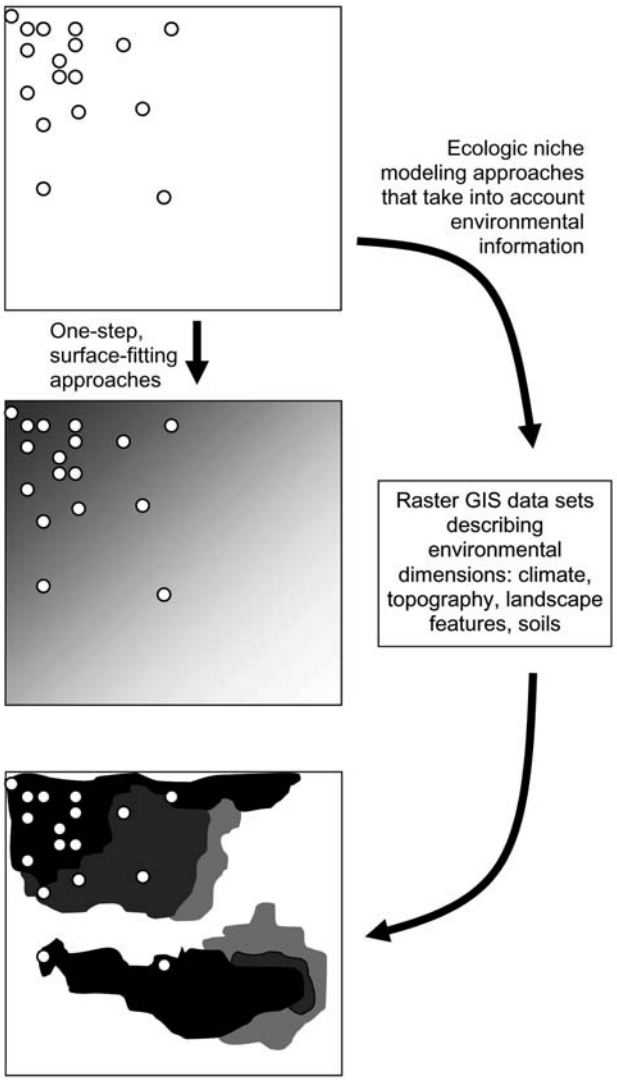

Figure. Hypothetical example of a species' known occurrences (circles) and inferences from that information. The middle panel shows the pattern that would result from a surface-fitting or smoothing algorithm, and the bottom panel shows the ability of ecologic niche modeling approaches to detect unknown patterns in biologic phenomena based on the relationship between known occurrences and spatial patterns in environmental parameters. GIS, geographic information system.

Understanding Ecology of Diseases

In many cases, the details of ecologic parameters associated with occurrences of diseases or of species participating in disease transmission (e.g., vectors, hosts, pathogens) may be unclear because of small sample sizes, biased reporting, or simply lack of detailed geographic or ecologic analysis. ENM encompasses a suite of tools that relate known occurrences of these species or phenomena to raster geographic information system layers that summarize variation in several environmental dimensions. The result is an objective, quantitative picture of how what is known about a species or phenomenon relates to environmental variation across a landscape. Studies using these approaches include an examination of ecologic differences among different Chagas disease vectors in Brazil (*11*) and a characterization of ecologic features of outbreaks of hemorrhagic fever caused by Ebola and Marburg viruses (*12*,*13*).

Characterizing Distributional Areas

A next step in applying ENM approaches to understanding disease systems is characterizing geographic distributions. Here, ENM (or something akin to it) is used to investigate landscapes for areas that meet the ecologic requirements of the species. The result is an interpolation between known sampling locations informed by observed associations between the species and environmental characteristics. Previous attempts to characterize geographic distributions of species in the disease realm have demonstrated the potential of the approach but have not always used the most powerful inferential techniques available (*14*,*15*). In at least 1 case (*14*), the methods used failed to generalize and predict into areas of sparse sampling. ENM produces statistically robust predictions of geographic distributions of species or phenomena (even in unsampled areas), greatly exceeding expectations under random (null) models. Numerous examples of applications of this functionality to disease systems have been published (*11*–*13*,*16*–*22*).

Identifying Areas of Potential Invasion in Other Regions

ENMs characterize general environmental regimes under which species or phenomena may occur. To the extent that the model is appropriately and correctly calibrated, it may be used to seek areas of potential distribution. Thus, ENMs can be used to identify areas that fit the ecologic bill for a species, even if the species is not present there. This approach has seen extensive experimentation and testing in the biodiversity realm (*8*,*23*), but applications to disease transmission have as yet been few. One study attempted to identify the particular species in the *Anopheles gambiae* complex that was responsible for the large-scale South American malaria outbreaks in the

early 20th century (*19*), and another evaluated the geographic potential of a possible monkeypox host (*Cricetomys* spp.) in North America (*24*).

Anticipating Risk Areas with Changing Climates

A logical extension of using ENMs to identify potential distributional areas is to address the question of likely geographic shifts in distributional areas of species or phenomena under scenarios of climate change or changing land use (*25*). This approach has seen considerable attention in the biodiversity realm, with both tests and validations (*26–28*), and with broad applications across faunas and floras (*29–32*). In the disease world, applications have been few, although 1 study used likely climate change–mediated range shifts to hypothesize the identity of *Lutzomyia* vectors of recent leishmaniasis outbreaks in southern Brazil (*21*).

Identifying Unknown Vectors or Hosts

ENM approaches can be applied to various parts of disease transmission cycles (e.g., overall case distribution, reservoir host distribution, vector distribution) to identify unknown elements in systems. The geography of overall case distributions can provide an indication of which clades are potential reservoirs and which are not. A first application was an attempt to identify mammalian hosts of the *Triatoma protracta* group of Chagas disease vectors in Mexico (*22*), which succeeded in anticipating the mammal hosts of 5 of 5 species for which a test was possible. Further exploration of this possible application of ENM methods has focused on the mysterious long-term reservoir of the filoviruses (Ebola and Marburg viruses) by comparing African mammal distributions with those of filovirus-caused disease outbreaks (*33*).

Discussion

Current Challenges in ENM

ENM, although it has old roots (*4*), is nonetheless a relatively new tool in distributional ecology and biogeography. Only a few recent studies have compared the performance of different methodologic approaches under the ENM rubric (*34–37*). As such, numerous challenges remain in terms of refining approaches toward a more powerful and synthetic methodology.

One central challenge is that of choosing modeling methods appropriate to a particular question, in the sense of discerning interpolation challenges from extrapolation challenges. In a recent comparative study focused on interpolation, which inferred details of patterns of presence and absence on a densely sampled landscape, several techniques that have internal controls on overfitting were superior (*34*). Extrapolative challenges, such as predicting potential distribution of invasive species, anticipating species' responses to global climate change, and identifying unknown reservoirs or vectors, require different qualities of modeling algorithms; different methods therefore appear to emerge as superior, according to the particular challenge (*5*). This balance of ability to interpolate accurately versus ability to extrapolate effectively remains a challenge for the ENM methods.

A second frontier that includes yet-to-be-resolved details for ENM is that of testing and evaluating model results. Currently accepted approaches center on the ability to predict independent test occurrence data in the smallest area predicted (*34*,*38*). However, efficient predictions can be poor descriptors of a species' geographic range. Simpler techniques that place greater emphasis on minimizing the omission of known occurrences may be more appropriate. Pairing significance tests (which demonstrate that the coincidence between a prediction and test data is better than that achieved by random or null models) with setting minimum performance criteria (which ensure that that the prediction is accurate enough to meet the needs of the study) is probably the best approach (*38*). However, these methods have yet to be agreed upon broadly in the ENM community.

Current Challenges in Applications of ENM to Disease Systems

Beyond methodologic challenges, several issues remain to be addressed for full application of ENM methods to disease systems. The first, and perhaps most important, is understanding the role of scale in space and time. Preliminary explorations suggest that proper matching of temporal and spatial scales in analyses may offer particular opportunities for precise and accurate prediction of the behavior of disease phenomena (*39*). Similarly, proper choice of environmental datasets requires further exploration. Climate data provide longer temporal applicability, but remotely sensed data that summarize aspects of surface reflectance can provide finer spatial resolution, and may measure aspects of ecologic landscapes that climate parameters alone may not capture (*40*). Such issues will be resolved only through further exploration and testing with predictive challenges for diverse disease systems.

Finally, because disease transmission systems often represent complex interactions among multiple species (e.g., vectors, hosts, pathogens), options exist for how they should be analyzed and modeled. Simple focus on disease occurrences, such as human cases, treats the entire transmission system as a black box and as such gives an overall picture of the ecology of the transmission chain of that disease (*12*). An alternative, however, is modeling each component species in the transmission system and then assembling the component ENMs into a geographic

picture of the transmission system (*22*). Each of these approaches has its relative advantages and disadvantages, but a best-practices method has yet to be established, pending further testing and exploration.

Conclusions

The emerging field of ENM applied to questions of ecologic and geographic characteristics of disease systems has considerable potential. In particular, it can solve several problems of spatial resolution of summaries of geographic risk for disease. In sharp contrast to surface-fitting approaches to the same questions, ENM does not lose resolution to generalize and produce a result. Rather, ENM can achieve fine-scale resolution of distributions limited only by the spatial precision of the input occurrence data and the input environmental datasets. This characteristic makes possible a clear improvement in the spatial resolution that is possible in representing spatial patterns in disease risk.

ENM is in the early stages of being explored for its potential for illuminating unknown phenomena in the world of disease transmission. The extensive explorations of ENM in the biodiversity field, however, serve as a benchmark of quality and acceptance for the technique. It can, once tested and prototyped extensively in the disease realm, offer a much-improved representation of spatial patterns in distributions of species or other phenomena.

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References

1. Waller LA, Carlin BP, Xia H, Gelfand AE. Hierarchical spatio-temporal mapping of disease rates. J Am Stat Assoc. 1997;92:607–17.
2. Kleinschmidt I, Bagayoko M, Clarke GPY, Craig M, Le Sueur D. A spatial statistical approach to malaria mapping. Int J Epidemiol. 2000;29:355–61.
3. MacNab YC, Dean CB. Autoregressive spatial smoothing and temporal spline smoothing for mapping rates. Biometrics. 2001;57:949–56.
4. Grinnell J. Field tests of theories concerning distributional control. Am Nat. 1917;51:115–28.
5. Soberón J, Peterson AT. Interpretation of models of fundamental ecological niches and species' distributional areas. Biodiversity Informatics. 2005;2:1–10.
6. Austin MP, Nicholls AO, Margules CR. Measurement of the realized qualitative niche: environmental niches of five *Eucalyptus* species. Ecol Monogr. 1990;60:161–77.
7. Guisan A, Zimmermann NE. Predictive habitat distribution models in ecology. Ecological Modelling. 2000;135:147–86.
8. Peterson AT. Predicting the geography of species' invasions via ecological niche modeling. Q Rev Biol. 2003;78:419–33.
9. Wiley EO, McNyset KM, Peterson AT, Robins CR, Stewart AM. Niche modeling and geographic range predictions in the marine environment using a machine-learning algorithm. Oceanography. 2003;16:120–7.
10. Soberón J, Peterson AT. Biodiversity informatics: managing and applying primary biodiversity data. Philos Trans R Soc Lond B Biol Sci. 2004;359:689–98.
11. Costa J, Peterson AT, Beard CB. Ecological niche modeling and differentiation of populations of *Triatoma brasiliensis* Neiva, 1911, the most important Chagas disease vector in northeastern Brazil (Hemiptera, Reduviidae, Triatominae). Am J Trop Med Hyg. 2002;67:516–20.
12. Peterson AT, Bauer JT, Mills JN. Ecologic and geographic distribution of filovirus disease. Emerg Infect Dis. 2004;10:40–7.
13. Peterson AT, Lash RR, Carroll DS, Johnson KM. Geographic potential for outbreaks of Marburg hemorrhagic fever. Am J Trop Med Hyg. 2006;75:9–15.
14. Rogers DJ, Randolph SE, Snow RW, Hay SI. Satellite imagery in the study and forecast of malaria. Nature. 2002;415:710–5.
15. Thomson MC, Elnaiem DA, Ashford RW, Connor SJ. Towards a kala azar risk map for Sudan: mapping the potential distribution of *Phlebotomus orientalis* using digital data of environmental variables. Trop Med Int Health. 1999;4:105–13.
16. Sánchez-Cordero V, Peterson AT, Martínez-Meyer E, Flores R. Distribución de roedores reservorios del virus causante del sindrome pulmonar por hantavirus y regiones de posible riesgo en México. Acta Zoologica Mexicana. 2005;21:79–91.
17. Peterson AT, Martínez-Campos C, Nakazawa Y, Martínez-Meyer E. Time-specific ecological niche modeling predicts spatial dynamics of vector insects and human dengue cases. Trans R Soc Trop Med Hyg. 2005;99:647–55.
18. Lopez-Cardenas J, González-Bravo FE, Salazar-Schettino PM, Gallaga-Solorzano JC, Ramírez-Barba E, Martínez-Mendez J, et al. Fine-scale predictions of distributions of Chagas disease vectors in the state of Guanajuato, Mexico. J Med Entomol. 2005;42:1068–81.
19. Levine RS, Peterson AT, Benedict MQ. Geographic and ecologic distributions of the *Anopheles gambiae* complex predicted using a genetic algorithm. Am J Trop Med Hyg. 2004;70:105–9.
20. Levine RS, Benedict MQ, Peterson AT. Distribution of *Anopheles quadrimaculatus* Say s.l. and implications for its role in malaria transmission in the US. J Med Entomol. 2004;41:607–13.
21. Peterson AT, Shaw JJ. *Lutzomyia* vectors for cutaneous leishmaniasis in southern Brazil: ecological niche models, predicted geographic distributions, and climate change effects. Int J Parasitol. 2003;33:919–31.
22. Peterson AT, Sánchez-Cordero V, Beard CB, Ramsey JM. Ecologic niche modeling and potential reservoirs for Chagas disease, Mexico. Emerg Infect Dis. 2002;8:662–7.
23. Skov F. Potential plant distribution mapping based on climatic similarity. Taxon. 2000;49:503–15.
24. Peterson AT, Papes M, Reynolds MG, Perry ND, Hanson B, Regnery R. Native-range ecology and invasive potential of *Cricetomys* in North America. Journal of Mammalogy. 2006;87:427–32.
25. Peterson AT, Tian H, Martínez-Meyer E, Soberón J, Sánchez-Cordero V, Huntley B. Modeling distributional shifts of individual species and biomes. In: Lovejoy TE, Hannah L, editors. Climate change and biodiversity. New Haven (CT): Yale University Press; 2005. p. 211–28.

26. Martínez-Meyer E, Peterson AT. Conservatism of ecological niche characteristics in North American plant species over the Pleistocene-to-recent transition. Journal of Biogeography. 2006;33:1779–89.
27. Martínez-Meyer E, Peterson AT, Hargrove WW. Ecological niches as stable distributional constraints on mammal species, with implications for Pleistocene extinctions and climate change projections for biodiversity. Global Ecology and Biogeography. 2004;13:305–14.
28. Araujo MB, Pearson RG, Thuiller W, Erhard M. Validation of species-climate impact models under climate change. Global Change Biology. 2005;11:1504–13.
29. Thuiller W, Lavorel S, Araujo MB, Sykes MT, Prentice IC. Climate change threats to plant diversity in Europe. Proc Natl Acad Sci U S A. 2005;102:8245–50.
30. Bakkenes M, Alkemade JR, Ihle F, Leemansand R, Latour JB. Assessing effects of forecasted climate change on the diversity and distribution of European higher plants for 2050. Global Change Biology. 2002;8:390–407.
31. Erasmus BFN, Van Jaarsveld AS, Chown SL, Kshatriya M, Wessels KJ. Vulnerability of South African animal taxa to climate change. Global Change Biology. 2002;8:679–93.
32. Peterson AT, Ortega-Huerta MA, Bartley J, Sánchez-Cordero V, Soberón J, Buddemeier RH, et al. Future projections for Mexican faunas under global climate change scenarios. Nature. 2002;416:626–9.
33. Peterson AT, Carroll D, Mills JN. Potential mammalian filovirus reservoirs. Emerg Infect Dis. 2004;10:2073–81.
34. Elith J, Graham CH, Anderson RP, Dudik M, Ferrier S, Guisan A, et al. Novel methods improve prediction of species' distributions from occurrence data. Ecography. 2006;29:129–51.
35. Manel S, Dias JM, Ormerod SJ. Comparing discriminant analysis, neural networks, and logistic regression for predicting species distributions: a case study with a Himalayan river bird. Ecological Modelling. 1999;120:337–47.
36. Stockwell DR, Peterson AT. Effects of sample size on accuracy of species distribution models. Ecological Modelling. 2002;148:1–13.
37. Stockwell DR, Peterson AT. Comparison of resolution of methods used in mapping biodiversity patterns from point occurrence data. Ecological Indicators. 2003;3:213–21.
38. Fielding AH, Bell JF. A review of methods for the assessment of prediction errors in conservation presence/absence models. Environmental Conservation. 1997;24:38–49.
39. Peterson AT, Martínez-Campos C, Nakazawa Y, Martínez-Meyer E. Time-specific ecological niche modeling predicts spatial dynamics of vector insects and human dengue cases. Trans R Soc Trop Med Hyg. 2005;99:647–55.
40. Roura-Pascual N, Suarez AV, McNyset K, Gómez C, Pons P, Wild TO, et al. Potential geographic distribution, ecological niche differentiation, and fine-scale regional projections for Argentine ants based on remotely-sensed data. Ecological Applications. 2006;16 (in press).

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Qualitative Assessment of Risk for Monkeypox Associated with Domestic Trade in Certain Animal Species, United States

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In 2003, US officials identified several human monkeypox cases and traced the virus exposure to infected captive prairie dogs. The virus was likely introduced through a shipment of imported African rodents, which were kept with other mammals, including prairie dogs, in a pet distribution facility in the Midwest. To prevent the further introduction and spread of the virus, federal agencies restricted the importation of African rodents and restricted the domestic trade or movement of prairie dogs and certain other rodents. In this qualitative assessment of the risk for monkeypox associated with the 2003 outbreak, we conclude that the probability of further human infection is low; the risk is further mitigated by rodent import restrictions. Were this zoonotic disease to become established domestically, the public health effects could be substantial.

In May and June 2003, public health officials identified an outbreak of human monkeypox in the United States (*1–3*). This was the first instance of human monkeypox virus (MPXV) infection detected outside its endemic range in Africa (*3*). As of July 30, 2003, a total of 72 human cases had been reported (*4*,*5*). Thirty-seven (51%) cases were eventually laboratory confirmed, and 35 met the case definition set by the Centers for Disease Control and Prevention (CDC) (*4*,*5*). Among the 35 patients whose cases were laboratory confirmed before July 11, 2003 (*1*), 32 (91%) tested positive for MPXV by PCR, culture, immunohistochemical testing, or electron microscopy of skin lesions; 2 tested positive by PCR and/or culture of an oropharyngeal or nasopharyngeal swab; and 1 tested positive by PCR and culture of a lymph node aspirate (*1*). To date, no new animal or human cases have been reported.

The outbreak was relatively large compared with most reported events in Africa, but clinical features were milder than typically seen there (*3*,*6*,*7*). No human deaths occurred (*1*,*8*), although 2 children required intensive care (*1*,*8*). One patient received a corneal transplant due to chronic ocular infection (*8*).

Most patients were exposed to prairie dogs, primarily from an Illinois animal distributor (IL-1). Most of those infected had direct physical contact with infected animals; infection likely resulted from bites or scratches or through open wounds (*1–3*). Some patients were exposed to premises where prairie dogs were kept (*1*).

Traceback implicated rodents from a shipment of African animals imported to Texas on April 9, 2003, as the probable source of MPXV (*1*,*4*). The shipment contained ≈800 small mammals of 9 different species, including 6 genera of African rodents (762 rodents total): rope squirrels, tree squirrels, Gambian giant rats, brushtail porcupines, dormice, and striped mice (*1*,*2*), as well as cusimanses, genets, and palm civets (*9*). Rodents from the shipment were housed with or in close proximity to prairie dogs at IL-1. Approximately 200 prairie dogs were at IL-1 coincident with the arrival of the imported African rodents (*1*). Many prairie dogs from IL-1 were distributed to other states for sale as pets (*1*,*4*). CDC traced 93 infected or potentially infected prairie dogs from IL-1 (*1*). An additional, unknown number of prairie dogs died or were sold at animal swap meets for which records are not available (*1*) (Table 1).

To prevent the introduction and spread of infected animals into susceptible populations, on June 11, 2003, the Food and Drug Administration (FDA) and CDC issued an order that prohibited 1) importation of all rodents from Africa and 2) transportation, sale, or any other commercial or public distribution, including release into the environment, of prairie dogs or rodents from 6 species represented in the African shipment (*10*). On November 4,

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Table 1. Disposition, as of July 11, 2003, of African rodents imported from Ghana to the United States on April 9, 2003*

Rodents	Dead†	Alive	Lost to follow-up	Total (n = 762)
Gambian giant rats	26	20	4	50
Dormice	≈350	27	≈135	≈510
Rope squirrels	49	4	–	53
Tree squirrels	24	20	3	47
Striped mice	14	50	36	100
Porcupines	2	–	–	2

*Source: (*1*).
†Includes animals that died of monkeypox and those that have been euthanized.

2003, FDA and CDC published an interim final rule (*11*) that imposed import restrictions on all African rodents and established or modified restrictions on the import, capture, transport, sale, barter, exchange, distribution, and release of prairie dogs, the 6 imported species, and possibly, by order, other animals with the potential to transmit MPXV. Neither CDC nor FDA exercised its statutory authority to seize and destroy animals to prevent the spread of MPXV.

We prepared this qualitative risk analysis to help understand the impact of the domestic trade restrictions on the current risk for human monkeypox infections. We evaluated the data and uncertainties concerning monkeypox and its potential spread to animal and human populations and characterized the probability of harm on the basis of those data. Because of CDC's import restrictions on all African rodents, we did not estimate the risk posed by importation of animals into the United States. We focused only on monkeypox and did not consider other zoonotic agents that might be transmitted by the species discussed. This risk assessment follows a generally accepted 4-part framework (*12*). The hazards are MPXV and its potential for transmission and spread from animals to humans; the risk is human infection from prairie dogs and possibly imported rodents.

Hazard Identification

Human monkeypox is a sporadic zoonotic viral disease, caused by an orthopoxvirus that until 2003 was known to have occurred only in parts of Africa (*3*,*7*,*13*). The first human illness was identified in 1970 in a child (*7*,*14*). Previous cases were likely mistaken for smallpox (*14*). Although it was first isolated from a captive primate (*3*,*6*), rodents are its likely primary natural reservoir (*7*,*15*–*17*); its complete mammalian host range is unknown. The mode of transmission between infected animals and humans is not well defined (*18*). Direct mucocutaneous contact and respiratory routes have been implicated in epidemiologic and experimental research (*15*,*18*,*19*).

The estimated mean human incubation period is 12 days (*1*,*3*,*15*,*16*). The disease is characterized by a rash similar to that observed with smallpox (*14*) or chickenpox (*10*,*15*,*20*). The infectious period occurs during the first week of the rash (*7*); symptoms include headache, fever, sweats, and severe lymphadenopathy (*15*,*16*,*20*). Among African patients with a history of smallpox vaccination, monkeypox is usually milder with lower numbers of deaths (*3*,*6*,*16*). Subclinical or very mild infection can occur in humans (*16*,*21*,*22*).

Case-fatality rates in African outbreaks range from 4% to 33% (*6*,*23*) and are high among children (*3*,*6*,*14*,*23*). Variability in case-fatality rates may reflect incomplete assessment of the total number of cases, variations in case definition, and variability in the virulence of MPXV strains. The US outbreak has been associated with a milder strain (*3*,*24*–*26*). Case fatality also likely depends on differences in exposure, susceptibility, and healthcare (*14*).

Repeated animal reintroduction of MPXV is believed necessary to endemic infections in human populations. Human cases in disease-endemic areas tend to be sporadic and isolated and primarily associated with direct animal-to-human transmission (*24*,*27*). However, clusters associated with common source and human-to-human transmission occur and may in Africa be increasing with decreased prevalence of prior smallpox vaccination (*6*,*7*,*15*,*16*,*28*,*29*).

FDA has not approved a treatment for monkeypox. Suggested treatment options include cidofovir (*30*–*32*). Efficacy of vaccinia immune globulin in humans has not been established (*30*,*31*). After the onset of symptoms, supportive therapy is usually the recommended treatment (*31*). Preexposure and postexposure smallpox vaccine was used during the 2003 outbreak, with only relatively minor adverse events reported (*1*,*32*).

Hazard Characterization

Much is unknown about pathogenesis and transmission dynamics of MPXV in humans and animals. Limited research suggests that at least in some host mammals latent or inapparent infection occurs (*15*). In addition to serologic evidence of orthopoxvirus exposure, MPXV has been recovered from the kidneys of healthy-appearing animals (*15*,*16*). The latency period is unknown, as is whether the virus can be transmitted during such periods.

The complete host range of MPXV in Africa is unknown. Animal antibody surveys in disease-endemic areas suggest infection is enzootic among squirrels, other

rodents, and monkeys, although other animals may be infected (*6*,*15*,*16*,*33*–*35*).

The number of animals exposed or infected in the United States is unknown and impossible to estimate. Approximately 800 animals were recorded in the African shipment, but disposition information is available only for rodents (Table 1) (*1*). A Gambian giant pouched rat, 3 dormice, and 2 rope squirrels from the shipment were tested and found to be infected with MPXV (*1*). Infected animals from the shipment were housed or transported with prairie dogs and other mammals. An unknown number of prairie dogs and animals from other species became infected. Although many prairie dogs became ill and several died, some infected animals survived. The secondary attack rate among susceptible animals is unknown and cannot be estimated with available data.

CDC necropsied 249 animals involved with the outbreak, confirming infection in 33 animals with PCR (*36*) and in 22 animals through virus isolation from various tissues. Infection was confirmed in 14 prairie dogs, 2 Gambian giant pouched rats, 9 dormice, 3 rope squirrels, 1 ground hog, 1 hedgehog, 1 jerboa, and 2 opossums.

CDC performed extensive histopathologic examination on 2 necropsied prairie dogs from IL-1 and detected MPXV DNA by using real-time PCR (*18*). The necropsied prairie dogs had MPXV in saliva, lesion exudates, and bronchi and lung parenchyma (*18*). Approximately 110 of the ≈200 prairie dogs likely exposed at IL-1 were sold after the African animals were introduced and before 15 of the prairie dogs at IL-1 became ill. Ten of the ill prairie dogs died rapidly (*1*,*3*,*18*).

In June 2003, CDC evaluated an unspecified number of prairie dogs, dormice, hedgehogs, jerboas, opossums, and numerous other species (a total of 18 species) from IL-1; of these, 2 prairie dogs, 7 dormice, 1 African hedgehog, 1 jerboa, and 1 gray short-tailed opossum tested positive for MPXV by PCR (*36*). When these animals were infected or if they could transmit disease is not known.

On June 19, 2003, CDC acquired 61 live animals from the original shipment. On August 20, 2003, CDC acquired from the state of Illinois 291 animals remaining at IL-1, including African and domestic species. Numerous other animals were acquired from Iowa, Wisconsin, Indiana, and Ohio. Of 172 animals tested from the various states as well as from the original shipment, 25 showed serologic evidence of infection without overt signs of disease (i.e., PCR and tissue culture negative). On June 24, 2003, an oral and ocular swab from a dormouse from IL-1 tested positive by PCR. After the dormouse died a month later, its tissues tested positive for MPXV by PCR and culture. A second dormouse from IL-1 that also tested positive in June appeared healthy; however, when it was euthanized in December 2003, swabs and necropsy samples of various tissues, urine, and feces were positive by PCR. No viral antigen was detected on pathologic examination of tissues (*36*).

Investigations of human cases from the outbreak support the hypothesis that close direct contact with infected animals was necessary for infection. Cases occurred among persons who were bitten by infected prairie dogs or infected through open wounds (*3*,*8*). The 11 Wisconsin patients included a child and parents; a meat distributor who also distributed exotic animals; his wife; 2 employees of 2 different pet stores; 2 veterinarians from different clinics; a person who had bought prairie dogs; and that person's houseguest. All of these patients reported direct contact with an infected prairie dog (*3*), although human-to-human transmission could not be ruled out for the parents (*3*).

Data on duration of infection are limited. Virus appears to be present in some animals months after infection, regardless of clinical illness. In addition to CDC's data on dormice, data derived from experimental infection of small numbers of laboratory animals documented infectious MPXV in tissues 3–6 weeks after exposure (*18*). Clinical and asymptomatic infections have been reported among captive primates; severity varied depending on the species and route of inoculation (*16*). CDC has reported elevated tissue viral loads in 2 necropsied prairie dogs (*18*). In another study, 10 experimentally infected North American ground squirrels died within 9 days, although no obvious signs of disease except for lethargy and anorexia developed (*37*). Squirrels infected intranasally had a longer incubation period and later death (*36*). Ten prairie dogs infected experimentally with a human MPXV isolate were highly susceptible to infection but had a lower death rate and less severe pathologic change than were seen in the squirrel study that used the same dose (*19*).

A human adult infected during the 2003 outbreak experienced keratitis and corneal ulceration as a complication of infection and ultimately received a corneal transplant (*8*). Corneal ulceration has also been reported in some African patients (*16*).

Exposure Assessment

In African outbreaks, capturing, handling, and eating wild animals have been associated with infection (*6*,*23*,*34*). In the United States, monkeypox occurred in humans who had direct contact with infected animals and were bitten or infected through open wounds (*3*). These persons included pet dealers, pet owners and their children, and contacts of these people at risk of coming into direct contact with the infected animal. Although potential exposure occurred in settings that included pet stores, swap meets, and wild animal trade centers (*1*), no evidence exists that persons casually exposed to infected animals

were infected. The magnitude and scope of this pet trade are not well quantified. In 2002, ≈30,000 prairie dogs were sold at pet dealers, swap meets, flea markets, and other venues open to the public (*11*).

Of the 762 rodents in the African shipment, CDC traced 584 (77%) (*1*). The remaining 178 (≈23%) could not be traced beyond the point of entry (*1*). The fate of the 50 nonrodent animals on the shipment is unknown. Of the ≈200 prairie dogs that may have been exposed to MPXV at IL-1, 107 (54%) have not been accounted for. These animals will not likely be traced. A small number of animals associated with the outbreak, including some known to have been infected, are in the possession of pet dealers and private owners; their capacity to transmit infection is unknown. Animals from species other than the listed species—gerbil, hamster, chinchilla, opossum, groundhog, hedgehog, and jerboa—were discovered to be infected, although no confirmed human cases of infection were associated with contact with any animal except prairie dogs (*1*,*9*).

To evaluate the potential spread of the disease beyond the initially exposed animals, the US Geological Survey's National Wildlife Health Center trapped 237 small mammals from 14 species at 9 sites in Wisconsin and Illinois where cases of monkeypox were reported. All were negative for monkeypoxvirus or monkeypoxvirus-specific antibodies (*38*). These small amounts of data are insufficient to establish the absence of MPXV in the wild.

The federal restrictions on importation of high-risk species and trade in the listed species have likely substantially reduced the potential risk for exposure of uninfected animals or persons to MPXV. However, some residual risk for MPXV infection through illegal importation or infection in legally imported, nonlisted species may exist.

Risk Characterization

Table 2 provides summary information on the qualitative variables considered in the risk characterization. We evaluated the probability of human monkeypox infection that resulted from certain types of exposure or contact (direct or indirect) to animals (infected or noninfected) and qualitatively estimated the probability and, to a lesser extent, the possible severity of infection. Most confirmed human cases in the United States were associated with direct, close contact with infected prairie dogs. We characterize as type I direct contact with the animal and as type II direct contact that also involves bites, scratches, or other contact with the mucous membranes or nonintact skin of the affected person. Infection through aerosolized particles without direct animal contact, or by some other less direct method, as well as human-to-human transmission, cannot be ruled out. The probability of infection is dependent on whether the animal is infectious (shedding virus) and varies with the level of shedding and the nature of human–animal interactions (type, frequency, and duration of contact). We assume, on the basis of the data described above, that the primary means of transmission affecting the risk would be from animal to human.

Several categories were established to define and qualitatively characterize the risks. Low risk denoted no direct

Table 2. Variables considered in characterizing risk for human monkeypox cases and the degree of uncertainty associated with these variables

Variable	Degree of uncertainty
Animal host and carrier species	High—some, but not all, host species identified
Proportion of probable host or carrier species infected with virus	High—need to assume absent data that all animals within known or probable carrier species are infected
Proportion of animals exposed during US 2003 outbreak infected with virus	High—need to assume that all exposed animals are infected
Susceptibility of naive animals to infection	High—but experience in United States and Africa suggests several species and orders can be infected with monkeypox virus
Latency in nonhuman species	High
Duration of infection or infectiousness in nonhuman species	High
Seasonality of disease	High—some indication of peak monkeypox cases in humans in July and August in African outbreaks, which may be associated with human behavior rather than characteristics of virus or host animals
Incubation in nonhuman species	High
Infection rates in exposed nonhuman species	High
Proportion of infected animals (of different species) that shed virus	High
Mode(s) of transmission across species and to humans	High—but evidence of mucocutaneous and respiratory transmission pathways
Attack rates among humans exposed to infected animals	High
Secondary attack rates among humans	High—secondary attack rates seem to be increasing in monkeypox-endemic areas due to increasing susceptibility of exposed populations, and historical data indicating low risk for human transmission may be unreliable
Fatality rates in nonhuman species	High

human contact with a captive animal(s); even if animal infection status was unknown or the animal was infected, the exposure to the animal was likely insufficient for animal-to-human disease transmission to occur. Medium risk described human contact that was direct but the exposure involved type I contact with a potentially infected animal or animal(s) of unknown infectious status. High risk designated direct human contact and involved a type II contact with a captive animal(s) infected with MPXV or with unknown infection status but likely MPXV exposure. Finally, the term severity of infection and illness denoted any individual infection with MPXV that should be considered serious and potentially fatal. The risk to the persons and the risk for the spread of the disease to others made MPXV infection a potentially serious public health matter.

The probability that any surviving animal directly involved in the outbreak may be infected must be considered high, given the possibility of latent infection. Making the unlikely assumption that all of these animals are still alive, the group includes the 178 African rodents, mostly dormice, lost to follow-up, 107 prairie dogs from IL-1 that were not traceable, and 50 nonrodent animals included in the African shipment. An unknown number, but clearly most, of the affected African rodents and IL-1 prairie dogs that were traced and identified as alive as of July 2003 (121 African rodents and ≈93 prairie dogs) have since died or been euthanized.

Some animals from other species that were in the affected pet distribution facilities during the outbreak tested positive for MPXV. An unknown number of these exposed animals are likely to be alive and in private or commercial ownership; what proportion of these animals is infected with MPXV is unknown but is assumed to be small. No confirmed cases of human infection or further cases of animal infection have been associated with these animals. However, all animals directly associated with the 2003 outbreak should be considered to pose a continued high risk for infection.

The probability of infection in rodents or other animals imported from monkeypox-endemic regions is unknown. Imported African rodents were almost certainly the source of the US outbreak. Animals imported as pets are handled by several persons as they pass from importer to owner, and they may be housed and transported in close proximity with nonimported susceptible animals. Current import restrictions on African rodents substantially reduce the risk for introduction and spread of MPXV, but a potential residual risk remains because of illegal importation as well as import of nonrestricted species that may carry the virus. Some previously imported animals from restricted species might also be infected with MPXV, although this risk is unknown and assumed to be extremely low.

For domestically bred African rodents, the risk they may pose of transmitting MPXV to humans depends on the risk that the rodents will be exposed to infected animals. Absent a tracking or pedigree system that distinguishes domestically bred from imported, wild-caught animals is impossible. Trade in domestically bred African rodents could increase the risk for human infection if illegally imported infected animals are identified as captive-bred. The monkeypox risk to humans posed by prairie dogs is a function of the animals' possible contact with infected animals and their potential for viral transmission.

The number of animals infected with or exposed to MPXV in the outbreak that might still be alive is likely small. However, these animals may be widely distributed geographically, and they may have spread the virus to other animals not currently known to pose a risk. The risk for MPXV infection and spread among prairie dogs are mitigated by current import and trade restrictions and the death or euthanization of most animals directly associated with the outbreak. The probability that an uninfected prairie dog will come into contact with an infected captive or released animal and that there will be sufficient exposure for infection is likely low. If such contact occurs, however, these animals are highly susceptible.

Little evidence about the MPXV status of wild prairie dogs exists. Given the high rates of illness and death among captive prairie dogs exposed to MPXV in 2003, anticipating that the virus would result in a die-off that would be detected may be reasonable; however, in addition to the lack of data, uncertainties about the virus and the susceptibility of the animals in the wild preclude drawing any conclusions.

The risk for new domestically acquired human cases is low with the current restrictions on import and trade in certain species in place. No new cases have been reported in humans or animals since the outbreak, despite the likelihood that some surviving infected animals have been kept alive by individual or commercial owners. Limited surveillance efforts have not identified MPXV in wild animal populations; however, the virus could possibly become enzootic here if an infected animal were released or escaped into the wild and spread the virus to susceptible mammals. Were that to occur, human cases would likely result. The risk that monkeypox could become enzootic is relevant in evaluating the risk of importing potential mammalian carriers of MPXV or in allowing contact between likely carriers and susceptible domestic mammals.

Data limitations preclude quantitative, and limit accurate qualitative, estimation of the human risk for monkeypox in the United States (Table 3). Research is needed on disease dynamics, range of host species, and the parameters of wild animal trade and ownership.

Table 3. Qualitative estimation of risk to humans*

Human exposure to animal	Animal infected	Infection status of animal unknown	
		Exposure likely	Exposure unlikely
Indirect exposure/no direct contact	Low	Low	Low
Direct contact, type I* (direct contact without type II exposure)	Medium	Medium	Low
Direct contact, type II* (bite, scratch, or contact of animal's body fluid with mucous membrane or nonintact skin)	High	High	Low

*Risk was based upon type of exposure to an animal and the infection status of the animal. Type I and type II are arbitrary classifications.

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References

1. Centers for Disease Control and Prevention. Update: multistate outbreak of monkeypox—Illinois, Indiana, Kansas, Missouri, Ohio, and Wisconsin, 2003. MMWR Morb Mortal Wkly Rep. 2003;52:642–6.
2. Centers for Disease Control and Prevention. Multistate outbreak of monkeypox—Illinois, Indiana, and Wisconsin, 2003. MMWR Morb Mortal Wkly Rep. 2003;52:537–40.
3. Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, Wegner MV, et al. The detection of monkeypox in humans in the Western Hemisphere. N Engl J Med. 2004;350:342–50.
4. Centers for Disease Control and Prevention. Monkeypox: report of cases in the United States. 2003. [cited 2006 Sept 26]. Available from http://www.cdc.gov/od/oc/media/mpv/cases.htm
5. Di Giulio DB, Eckburg PB. Human monkeypox. Lancet Infect Dis. 2004;4:199.
6. Hutin YJ, Williams RJ, Malfait P, Pebody R, Loparev VN, Ropp SL, et al. Outbreak of human monkeypox, Democratic Republic of Congo, 1996 to 1997. Emerg Infect Dis. 2001;7:434–8.
7. Huhn GD, Bauer AM, Yorita K, Graham MB, Sejvar J, Likos A, et al. Clinical characteristics of human monkeypox, and risk factors for severe disease. Clin Infect Dis. 2005;41:1742–51.
8. Di Giulio DB, Eckburg PB. Human monkeypox: an emerging zoonosis. Lancet Infect Dis. 2004;4:15–25.
9. Reynolds MG, Cono J, Curns A, Holman RC, Likos A, Regnery R, et al. Human monkeypox. Lancet Infect Dis. 2004;4:604–5.
10. Gerberding JL, McClellan MB. Joint Order of the Centers for Disease Control and Prevention and the Food and Drug Administration, Department of Health and Human Services. June 11, 2003. Available at 68 Fed Reg. 36566 (June 18, 2003).
11. Centers for Disease Control and Prevention. Control of communicable diseases; restrictions on African rodents, prairie dogs, and certain other animals. Interim final rule. Fed Regist. 2003;68:62353–69.
12. National Research Council. Risk assessment in the federal government: managing the process. Washington: National Academy Press; 1983.
13. Ladnyj ID, Ziegler P, Kima E. A human infection caused by monkeypox virus in Basankusu Territory, Democratic Republic of the Congo. Bull World Health Organ. 1972;46:593–7.
14. Jezek Z, Szczeniowski M, Paluku KM, Mutombo M. Human monkeypox: clinical features of 282 patients. J Infect Dis. 1987;156:293–8.
15. Breman JG. Monkeypox: an emerging infection for humans? In: Scheld WM, Craig WA, Hughes JM, editors. Emerging Infections 4. Washington: ASM Press; 2000. p. 45–76.
16. Jezek Z, Fenner F. Human monkeypox, in Monographs on Virology. New York: Karger; 1988.
17. Khodakevich L, Szczeniowski M, Manbu-ma-Disu, Jezek Z, Marennikova S, Nakano J, et al. The role of squirrels in sustaining monkeypox virus transmission. Trop Geogr Med. 1987;39:115–22.
18. Guarner J, Johnson BJ, Paddock CD, Shieh WJ, Goldsmith CS, Reynolds MG, et al. Monkeypox transmission and pathogenesis in prairie dogs. Emerg Infect Dis. 2004;10:426–31.
19. Xiao SY, Sbrana E, Watts DM, Siirin M, da Rosa AP, Tesh RB. Experimental infection of prairie dogs with monkeypox virus. Emerg Infect Dis. 2005;11:539–45.
20. Anderson MG, Frenkel LD, Homann S, Guffey J. A case of severe monkeypox virus disease in an American child: emerging infections and changing professional values. Pediatr Infect Dis J. 2003;22:1093–6.
21. Jezek Z, Nakano JH, Arita I, Mutombo M, Szczeniowski M, Dunn C. Serological survey for human monkeypox infections in a selected population in Zaire. J Trop Med Hyg. 1987;90:31–8.
22. Jezek Z, Marennikova SS, Mutumbo M, Nakano JH, Paluku KM, Szczeniowski M. Human monkeypox: a study of 2,510 contacts of 214 patients. J Infect Dis. 1986;154:551–5.
23. Meyer H, Perrichot M, Stemmler M, Emmerich P, Schmitz H, Varaine F, et al. Outbreaks of disease suspected of being due to human monkeypox virus infection in the Democratic Republic of Congo in 2001. J Clin Microbiol. 2002;40:2919–21.
24. Zaucha GM, Jahrling PB, Geisbert TW, Swearengen JR, Hensley L. The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (*Macaca fascicularis*). Lab Invest. 2001;81:1581–600.
25. Esposito JJ, Knight JC. Orthopoxvirus DNA: a comparison of restriction profiles and maps. Virology. 1985;143:230–51.
26. Likos AM, Sammons SA, Olson VA, Frace AM, Li Y, Olsen-Rasmussen M, et al. A tale of two clades: monkeypox viruses. J Gen Virol. 2005;86:2661–72.
27. Arita I, Jezek Z, Khodakevich L, Ruti K. Human monkeypox: a newly emerged orthopoxvirus zoonosis in the tropical rain forests of Africa. Am J Trop Med Hyg. 1985;34:781–9.
28. Heymann DL, Szczeniowski M, Esteves K. Re-emergence of monkeypox in Africa: a review of the past six years. Br Med Bull. 1998;54:693–702.

29. Antia R, Regoes RR, Koella JC, Bergstrom CT. The role of evolution in the emergence of infectious diseases. Nature. 2003;426:658–61.
30. Centers for Disease Control and Prevention. Updated interim CDC guidance for use of smallpox vaccine, cidofovir, and vaccinia immunoglobulin (VIG) for prevention and treatment in the setting of an outbreak of monkeypox infections. 2003. [cited 2006 Sep 26]. Available from www.cdc.gov/ncidod/monkeypox/treatmentguidelines.hrm
31. Sejvar JJ, Chowdary Y, Schomogyi M, Stevens J, Patel J, Karem K, et al. Human monkeypox infection: a family cluster in the midwestern United States. J Infect Dis. 2004;190:1833–40.
32. Edghill-Smith Y, Golding H, Manischewitz J, King LR, Scott D, Bray M, et al. Smallpox vaccine–induced antibodies are necessary and sufficient for protection against monkeypox virus. Nat Med. 2005;11:740–7.
33. Centers for Disease Control and Prevention. Human monkeypox—Kasai Oriental, Democratic Republic of Congo, February 1996–October 1997. MMWR Morb Mortal Wkly Rep. 1997;46:1168–71.
34. Khodakevich L, Jezek Z, Messinger D. Monkeypox virus: ecology and public health significance. Bull World Health Organ. 1988;66:747–52.
35. Breman JG, Nakano JH, Coffi E, Godfrey H, Gautun JC. Human poxvirus disease after smallpox eradication. Am J Trop Med Hyg. 1977;26:273–81.
36. Hutson CL, Lee KN, Abel J, Carroll DS, Montgomery JM, Olson VA, et al. Monkeypox zoonotic association: insights from laboratory evaluation of animals associated with the U.S. outbreak. Am J Trop Med Hyg. 2006; (in press).
37. Tesh RB, Watts DM, Sbrana E, Siirin M, Popov VL, Xiao S-Y. Experimental infection of ground squirrels (*Spermophilus tridecemlineatus*) with monkeypox virus. Emerg Infect Dis. 2004;10:1563–7.
38. US Department of the Interior; US Geological Survey. Investigating monkeypox in the wild. Factsheet 2004–3003. July 2004. [cited 2006 Jun 13]. Available from http://www.nwhc.usgs.gov/publications/fact_sheets/pdfs/monkeypoxfinal_071504.pdf

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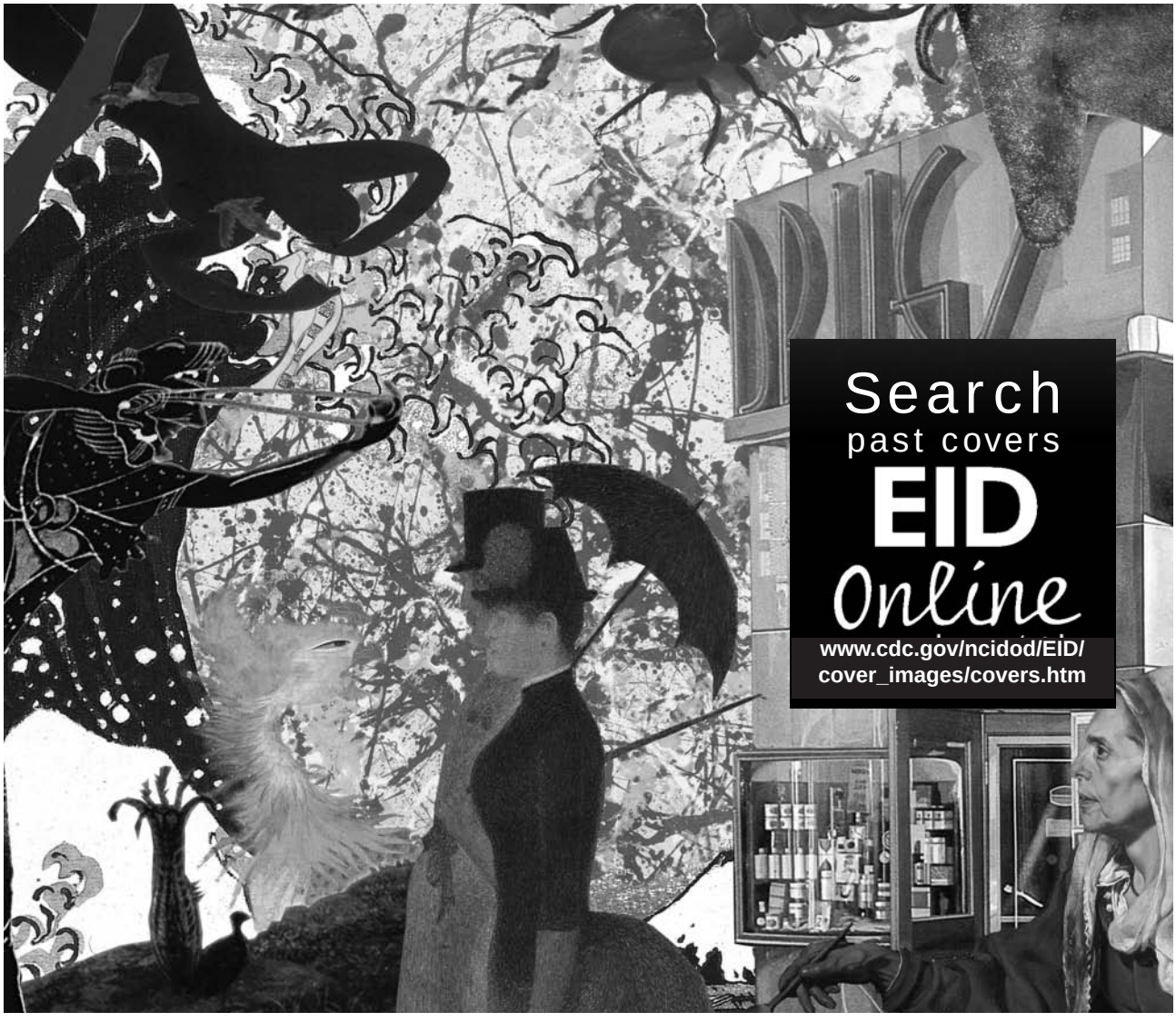

Review of Bats and SARS

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Bats have been identified as a natural reservoir for an increasing number of emerging zoonotic viruses, including henipaviruses and variants of rabies viruses. Recently, we and another group independently identified several horseshoe bat species (genus *Rhinolophus*) as the reservoir host for a large number of viruses that have a close genetic relationship with the coronavirus associated with severe acute respiratory syndrome (SARS). Our current research focused on the identification of the reservoir species for the progenitor virus of the SARS coronaviruses responsible for outbreaks during 2002–2003 and 2003–2004. In addition to SARS-like coronaviruses, many other novel bat coronaviruses, which belong to groups 1 and 2 of the 3 existing coronavirus groups, have been detected by PCR. The discovery of bat SARS-like coronaviruses and the great genetic diversity of coronaviruses in bats have shed new light on the origin and transmission of SARS coronaviruses.

Severe acute respiratory syndrome (SARS) represents the 21st century's first pandemic of a transmissible disease with a previously unknown cause. The pandemic started in November 2002 and was brought under control in July 2003, after it had spread to 33 countries on 5 continents, resulting in >8,000 infections and >700 deaths (*1*). The outbreaks were caused by a newly emerged coronavirus, now known as the SARS coronavirus (SARS-CoV).

In late 2003 and early 2004, sporadic outbreaks were reported in the region of the People's Republic of China where the 2002–2003 outbreaks originated (*2*). However, molecular epidemiologic studies showed that the viruses responsible for the 2003–2004 outbreaks were not the same as those isolated during the 2002–2003 outbreaks (*3*). These findings indicate independent species-crossing events. They also indicate that a SARS epidemic may recur in the future and that SARS-like coronaviruses (SARS-like–CoVs) that originate from different reservoir host populations may lead to epidemics at different times or in different regions, depending on the distribution of the reservoirs and transmitting hosts. The recent discovery of a group of diverse SARS-like–CoVs in bats supports the possibility of these events and further highlights the need to understand reservoir distribution and transmission to prevent future outbreaks.

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Animal Origin of SARS Coronaviruses

Because of the sudden and unpredictable nature of the SARS outbreaks that started in November 2002 in southern People's Republic of China, structured and reliable epidemiologic studies to conclusively trace the origin of SARS-CoV were not conducted. However, accumulated studies from different groups, which used a variety of approaches, indicated an animal origin on the basis of the following findings. 1) Genome sequencing indicated that SARS-CoV is a new virus with no genetic relatedness to any known human coronaviruses (*4*,*5*). 2) Retrospective serologic studies found no evidence of seroprevalence to SARS-CoV or related viruses in the human population (*6*). 3) Serologic surveys among market traders during the 2002–2003 outbreaks showed that antibodies against SARS-CoV or related viruses were present at a higher ratio in animal traders than control populations (*7*–*9*). 4) Epidemiologic studies indicated that early case-patients were more likely than later case-patients to report living near a produce market but not near a farm, and almost half of them were food handlers with probable animal contact (*7*). 5) SARS-CoVs isolated from animals in markets were almost identical to human isolates (*9*). 6) Molecular epidemiologic analyses indicated that human SARS-CoV isolates could be divided into 3 groups from the early, middle, and late phases of the outbreaks and that early-phase isolates were more closely related to the animal isolates (*10*). 7) Human SARS-CoVs isolates from the 2003–2004 outbreaks had higher sequence identity to animal isolates of the same period than to human isolates from the 2002–2003 outbreaks (*3*).

Susceptible Animals in Markets and Laboratories

The first evidence of SARS-CoV infection in animals came from a study conducted in a live animal market in early 2003 (*9*). From the 25 animals sampled, viruses closely related to SARS-CoV were detected in 3 masked palm civets (*Paguma larvata*) and 1 raccoon dog (*Nyctereutes procyonoides*). In addition, neutralizing antibodies against SARS-CoV were detected in 2 Chinese ferret badgers (*Melogale moschata*). This initial study indicated that at least 3 different animal species in the Shenzhen market were infected by coronaviruses that are closely related to SARS-CoV.

Given the vast number of live animals being traded in animal markets in southern People's Republic of China, knowing which other animals are also susceptible to these viruses is crucial. Unfortunately, for a variety of reasons no systematic studies were conducted on traded animals during the outbreak period. Experimental infection of different animals therefore became a component of the SARS-CoV investigation.

Currently, >10 mammalian species have been proven to be susceptible to infection by SARS-CoV or related viruses (Table 1). Rats were also implicated as potentially susceptible animals that may have played a role in the transmission and spread of SARS-CoV in the well-publicized SARS outbreaks in the Amoy Gardens apartment block in Hong Kong Special Administrative Region, People's Republic of China (*23*). In Guangdong in 2004, the first human with a confirmed case of SARS was reported to have had no contact with any animals except rats (*2*). Experimentally, we have obtained serologic evidence that SARS-CoV replicates asymptomatically in rats (B.T. Eaton et al., unpub. data). Further studies are needed to clarify the potential role of rats in the transmission of SARS-CoV. Studies by 2 independent groups suggested that avian species were not susceptible to SARS-CoV infection and that, hence, domestic poultry were unlikely to be the reservoir or associated with the dissemination of SARS-CoV in the animal markets of southern People's Republic of China (*22*,*24*).

Role of Masked Palm Civets

Although in 1 live animal market, 3 species were found to be infected by viruses related to SARS-CoV (*9*), all subsequent studies have focused mainly on palm civets, possibly because the rate of detection was higher in civets or because the number of civets traded in southern People's Republic of China exceeds that of other wildlife groups.

The isolation of closely related SARS-CoV in civets during the 2002–2003 and 2003–2004 outbreaks and the close match of virus sequences between the human and civet isolates from each outbreak (*3*,*9*,*25*) strongly suggest that civets are a direct source of human infection. However, these studies did not clarify whether animals other than civets were involved in transmission of SARS-CoV to humans or whether civets were an intermediate host or the natural reservoir host of SARS-CoVs.

During the 2002–2003 outbreaks, none of the animal traders surveyed in the markets, who supposedly had very close contact with live civets, displayed SARS symptoms

Table 1. Animal species susceptible to infection by SARS coronavirus*

Animal		Mode of infection	Clinical signs	References
Common name	Taxonomic name			
Masked palm civet	*Paguma larvata*	Natural	None observed	(*9*)
		Experimental	Fever, lethargy, reduced appetite	(*11*)
Racoon dog	*Nyctereutes procyonoides*	Natural	None observed	(*9*)
Chinese ferret badger	*Melogale moschata*	Natural	None observed	(*9*)
Cynomolgus macaque	*Macaca facicularis*	Experimental	Lethargy, skin rash, respiratory distress	(*12*)
Rhesus macaque	*Macaca mulatta*	Experimental	Fever, low appetite	(*13*,*14*)
African green monkey	*Cercopithecus aethiops*	Experimental	None observed	(*15*)
Ferret	*Mustela furo*	Experimental	Lethargy, mild pulmonary lesions	(*16*)
Golden hamster	*Mesocricetus auratus*	Experimental	None observed	(*17*)
Guinea pig	*Cavia porcellus*	Experimental	None observed	(*18*)
Mouse	*Mus musculus*	Experimental	Aged animal (12–14 mo): weight loss, hunched posture, ruffled fur, slight dehydration	(*19*)
			Young animal (4–6 weeks): none observed	(*20*)
Rat	*Rattus rattus*	Experimental	None observed	B.T. Eaton et al., unpub. data
Domestic cat	*Felis domesticus*	Natural	Not reported	(16)
		Experimental	None observed	(*16*)
Pig	*Sus scrofa*	Natural	Not reported	(*21*)
		Experimental	None observed	(*22*)

*SARS, severe acute respiratory syndrome.

(*7*–*9*). During the 2003–2004 outbreaks, at least 1 human SARS patient had had no contact with civets (*2*). These observations seem to indicate that ≥1 other animal species may play a role in transmission of SARS-CoV to humans.

Most, if not all, civets traded in the markets are not truly wildlife animals; rather, they are farmed animals. Civet farming is relatively new in People's Republic of China and has rapidly expanded during the past 15 years or so. Tu et al. conducted the first comparative study of market and farmed civets (*26*). Serologic testing was performed on 103 serum samples taken from civets in an animal market in Guangdong and several civet farms in different regions of People's Republic of China in June 2003 and January 2004. No significant level of SARS-CoV antibody was detected in any of the 75 samples taken from 6 farms in 3 provinces. In contrast, of the 18 samples taken from an animal market in Guangdong Province in January 2004, 14 (79%) had neutralizing antibodies to SARS-CoV.

In a parallel study conducted between January and September 2004 (*27*), molecular analysis was used to investigate the distribution of SARS-CoV in palm civets in markets and on farms. PCR analysis of samples from 91 palm civets and 15 raccoon dogs in 1 animal market and 1,107 civets from 25 farms in 12 provinces showed positive results for all animals from the market and negative results for all animals from the farms. Similar results were obtained in wild-trapped civets in Hong Kong; none of the 21 wild civets sampled had positive antibody or PCR results for SARS-CoV (*28*).

Although not universally true, natural reservoir hosts tend to have coevolved with their viruses and usually do not display clinical signs of infection (*29*). However, when palm civets were experimentally infected with 2 strains of human SARS-CoV, all developed clinical signs of fever, lethargy, and loss of aggressiveness (*11*).

Civits' high susceptibility to SARS-CoV infection and wide presence in markets and restaurants strongly indicates an important role for civets in the 2002–2003 and 2003–2004 SARS outbreaks. However, the lack of widespread infection in wild or farmed palm civets makes them unlikely to have been the natural reservoir host.

SARS-like Coronaviruses in Bats

The presence of SARS-like–CoVs in different species of horseshoe bats in the genus *Rhinolophus* has recently been reported. We found, in a study of horseshoe bat species in different regions of mainland People's Republic of China in 2004 (*30*), that each of the 4 species surveyed had evidence of infection by a SARS-like–CoV: 2 species (*R. pearsoni* and *R. macrotis*) had positive results by both serologic and PCR tests, and 2 (*R. pussilus* and *R. ferrumequinum)* had positive results by either serologic or PCR tests, respectively. Bats with positive results were detected in the provinces of Hubei and Guangxi, which are >1,000 km apart. A group in Hong Kong (*31*) found that, when analyzed by PCR, 23 (39%) of 59 anal swabs of wild Chinese horseshoe bats (*R. sinicus*) contained genetic material closely related to SARS-CoV. They also found that as many as 84% of the horseshoe bats examined contained antibodies to a recombinant N protein of SARS-CoV. A previous study indicated a certain level of antigenic cross-reactivity between SARS-CoV and some group 1 coronaviruses (*6*) and that several group 1 coronaviruses had recently been found in bats. Therefore, the actual seropositive proportion of *R. sinicus* might be <84%. Nevertheless, the relatively high seroprevalence and wide distribution of seropositive bats is consistent with the serologic pattern expected from a pathogen's natural reservoir host (*29*).

Genome sequencing showed that the genome organization of all bat SARS-like–CoVs is almost identical to that of the SARS-CoVs isolated from humans or civets. They shared an overall sequence identity of 88% to 92%. The most variable regions were located in the 5′ end of the S gene, which codes for the S1 domain responsible for receptor binding, and in open reading frame 10 (ORF10 or ORF8, depending on the nomenclature used) region immediately upstream from the N gene (Figure, panel A, region b), which is known to be also prone to deletions of various sizes (*3*,*9*). Most human SARS-CoVs isolated during the late phase of the 2002–2003 outbreaks have a 29-nt deletion in this region; this deletion is absent in the civet isolates or human isolates from the early phase of the outbreaks (*3*,*9*). The bat viruses also lack the 29-nt deletion, indicating that SARS-CoVs and SARS-like–CoVs share a common ancestor.

Furthermore, sequence analyses indicated the existence of a much greater genetic diversity of SARS-like–CoVs in bats than of SARS-CoVs in civets or humans, which supports the notion that SARS-CoV is a member of this novel coronavirus group and that bats are a natural reservoir for it. The overall genome sequence identities between the human/civit SARS-CoVs and the bat viruses Rp3 (isolated from *R. pearsoni*) and HKU3–1 (isolated from *R. sinicus*) were 92% and 88%, respectively. The sequence identity between the bat isolates Rp3 and HKU3–1 is 89%, which indicates that the genetic divergence among the bat isolates is as great as the divergence between each of the bat viruses and the human/civet isolates. In addition, phylogenetic trees based on different protein sequences display different tree topologies, as shown in the Figure (panel B), which indicates the existence of multiple evolutionary pathways for different regions of the genome.

Diversity of Coronaviruses in Bats

The discovery of SARS-CoV has boosted the search for novel coronaviruses of human and animal origin. Bats

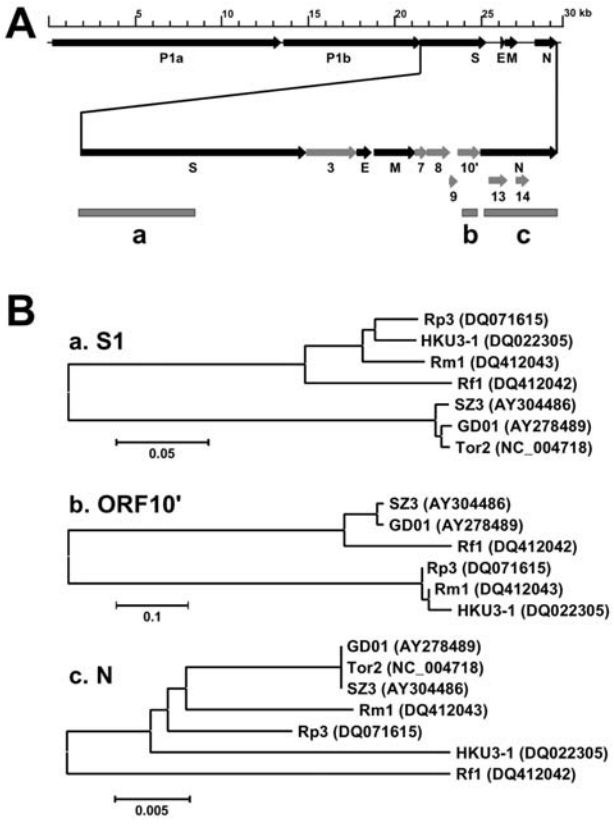

Figure. A) Genome diagram indicating the location of structural (dark arrow) and nonstructural (shaded arrow) genes and the different regions (shaded boxes) used for phylogeny analysis. B) Phylogenetic trees based on deduced amino acid sequences of the spike protein S1 domain (a), the open reading frame (ORF)10' (b), and the N protein (c). Because of lack of the ORF10' coding region in Tor2, Tor2 could not be included for the tree in (b). GD01, human isolate from early phase of the outbreak in 2003; Tor2, human isolate from late phase of the outbreak in 2003; SZ3, civet isolate from March 2003; Rp3, bat isolate from *Rhinolophus pearsoni*, December 2004; Rf1, bat isolate from *R. ferrumequinum*, November 2004; Rm1, bat isolate from *R. macrotis*, November 2004; and HKU3-1, bat isolate from *R. sinicus*, February 2005. GenBank accession nos. appear next to isolate names.

have been chosen as the main target because of their species diversity, large population size, broad geographic distribution, ability for long-distance migration, and habit of roosting in large groups. In addition to the SARS-like–CoVs described above, many other coronaviruses have been detected by PCR among diverse bat populations in Hong Kong (Table 2).

Poon et al. (*28*) conducted a surveillance study in Hong Kong during the summers of 2003 and 2004. From 162 swab samples collected from 12 bat species, they detected a novel group 1 coronavirus by sequencing of PCR products from the RNA-dependent RNA polymerase (RdRp) gene. The same virus or viruses of the same genetic lineage were found in 3 *Miniopterus* species (*M. pusillus, M. magnater,* and *M. schreibersii*). However, attempts to isolate virus by using 3 different cell lines (MDCK, FRhK4, and VeroE6) were unsuccessful.

In another study in Hong Kong during April 2004–July 2005, Woo et al. (*32*) sampled 309 individual bats representing 13 species from 20 different locations in rural Hong Kong. They detected coronavirus-related viral genomic RNA in 37 bats, representing a prevalence of 12%. Partial sequencing of *RdRp* and helicase genes identified 8 coronavirus genome types, 2 of which were the same as those reported previously (*28*,*30*,*31*). The other 6 novel types of coronaviruses were obtained from 6 different bat species and phylogenetically positioned in 2 of the existing 3 coronavirus groups. Four were in group 1, derived from bat species *M. magnater, M. pusillus, Myotis ricketti,* and *R. sinicus;* the other 2 were in group 2, from bat species *Pipistrellus abramus* and *Tylonycteris pachypus.* To accommodate the newly discovered genetic diversity of group 2 coronaviruses, the authors proposed the following subdivisions: group 2a (coronaviruses existing before the discovery of SARS-CoV), group 2b (SARS-CoV and SARS-like–CoVs), and group 2c (novel bat coronaviruses discovered in this study). Attempts to isolate virus in VeroE6, MRC-5, LLC-Mk2, FRhK-4, Huh-7.5, and HRT-18 were unsuccessful.

In another extensive study conducted in mainland People's Republic of China during November 2004–March 2006, Tang et al. (*33*) collected samples from 985 bats: 35 species in 14 genera and 3 families at 82 different sites in 15 provinces. A total of 64 (6.5%) bats had positive results from a PCR directed to a highly conserved 440-bp RdRp region. Among the 64 PCR-positive products sequenced, only 3 (all from the genus *Rhinolophus*) were clustered with known bat SARS-like–CoVs (or group 2b), 40 belonged to group 1, and the remaining 22 formed a separate cluster in group 2, most likely clustering with the group 2c viruses reported by Woo et al. (*32*). Attempts to isolate virus in VeroE6, FRHK4, and CV1 were unsuccessful.

In addition to the diversity of coronaviruses in bats, 3 more observations can be drawn from these studies. First, none of the bat coronaviruses discovered so far belonged to group 3. Second, with very few exceptions, most bat coronaviruses seem to be species-specific; i.e., different bat species from a similar location harbor different coronaviruses, whereas the same bat species from different geographic locations carry coronaviruses of the same genetic lineage (*32*,*33*). Third, among the 5 published studies involving bat coronaviruses (*28*,*30*–*33*), no researchers were able to isolate live virus from any of the swab samples collected despite the use of many different cell lines

Table 2. Coronaviruses detected in different species of bats

Viruses		Bat species	Location of detection (People's Republic of China)	Reference
Group*	Name/strain			
G1	Bat-CoV HKU2	*Rhinolophus sinicus*	Hong Kong	(*32*)
	Bat-CoV HKU6	*Myotis ricketti*	Hong Kong	(*32*)
	Bat-CoV HKU7	*Miniopterus magnater*	Hong Kong	(*32*)
	Bat-CoV HKU8	*Miniopterus pusillus* *M. magnater* *Miniopterus schreibersii*	Hong Kong	(*28*,*32*)
	BtCoV/701/05	*Myotis ricketti*	Anhui, Yunnan, Guangdong	(*33*)
	BtCoV/821/05	*Myotis ricketti*	Jiangxi, Guangxi	(*33*)
	BtCOV/821/05	*Scolophlus kuhlii*	Hainan	(*33*)
	BtCoV/970/06	*Rhinolophus pearsoni* *Rhinolophus ferrumequinum*	Shandong	(*33*)
	BtCoV/A773/05	*M. schreibersii*	Fujian	(*33*)
	BtCoV/A011/05	*M. schreibersii*	Anhui, Fujian, Guangxi	(*33*)
G2b	Rp3	*R. pearsoni*	Guangxi	(*30*)
	Rm1 (BtCoV/279/04)	*Rhinolophus macrotis*	Hubei	(*30*)
	Rf1 (BtCoV/273/04)	*R. ferrumequinum*	Hubei	(*30*)
	Bat-SARS-CoV HKU3	*R. sinicus*	Hong Kong	(*31*,*32*)
	BtCoV/A1018/06	*R. sinicus*	Shandong	(*33*)
	BtCoV/279/04	*R, macrotis*	Hubei	(*33*)
	BtCoV/273/04	*R. ferrumequinum*	Hubei	(*33*)
G2c	Bat-CoV HKU4	*Tylonycteris pachypus*	Hong Kong	(*32*)
	Bat-CoV HKU5	*Pipistrellus abramus*	Hong Kong	(*32*)
	BtCoV/133/05	*T. pachypus*	Guangdong	(*33*)
	BtCoV/434/05	*Pipistrellus pipistrellus*	Hainan	(*33*)
	BtCoV/355/05	*P. abramus* *R. ferrumequinum*	Anhui, Henan, Sichuan	(*33*)

*Group 2 was subdivided as in (*32*). A different classification was used in (*33*), in which G2b and G2c were designated G4 and G5, respectively.

and the presence of high levels of viral genetic materials shown by quantitative PCR.

Cross-species Transmission

Emergence of zoonotic viruses from a wildlife reservoir requires 4 events: 1) interspecies contact, 2) cross-species virus transmission (i.e., spillover), 3) sustained transmission, and 4) virus adaptation within the spillover species (*34*). These 4 transition events occurred during the SARS outbreaks and contributed to the rapid spread of the disease around the world.

The role of civets in directly transmitting SARS-CoV to humans has been well established. The most convincing case was the infection of a waitress and a customer in a restaurant where SARS-CoV–positive civets were housed in cages (*25*). Two key questions remain: What is the natural reservoir host for the outbreak SARS-CoV strains, and how were the viruses transmitted to civets or other intermediate hosts? Although not conclusive, the data obtained so far strongly suggest that bats (horseshoe bats in particular) are most likely the reservoir host of SARS-CoV. As indicated above, bat coronaviruses seem to be species-specific and SARS-like–CoVs discovered so far are exclusively associated with horseshoe bats. We hope that continued field study will eventually identify the direct progenitor of SARS-CoV among the 69 different known horseshoe species. The facts that the cross-species transmission of SARS-CoV seems to be a relatively rare event and that legal and illegal trading of wildlife animals between People's Republic of China and other countries occurs raise the possibility that the natural reservoir species may not be native to People's Republic of China. Thus, we should expand our search into regions other than Hong Kong and mainland People's Republic of China. Another approach to search for the natural reservoir of SARS-CoV is to conduct infection experiments in different bat species. If we assume that the progenitor viruses come from bats, chances are high that the human/civet SARS-CoVs are still capable of infecting the original reservoir species.

Without knowing the natural reservoir of SARS-CoV, predicting the exact mechanism of transmission from reservoir host to intermediate host is difficult. However, the fecal-oral route represents the main mode of transmission among animals. Although mixing of live reservoir hosts (e.g., bats) and intermediate hosts (e.g., civets) would be an efficient means of transmission, the main source of cross-species transmission in the animal trading chain (including warehouses, transportation vehicles, markets) may come from contaminated feces, urine, blood, or aerosols. This may also be true for civet-to-human transmission. As shown in the case of the infected restaurant

customer in 2004, the customer had no direct contact with civets and was sitting at a table ≈5 m from the civet cages (*25*).

Although at this stage we cannot rule out the possibility of direct transmission from the natural reservoir host to humans, molecular epidemiologic studies (*2*,*10*) and studies of the receptor-S protein interaction (*35*) indicate that the progenitor viruses are unlikely to be able to infect humans and that a rapid viral evolution in an intermediate host (such as civets) seems to be necessary to adapt the virus for human infection. Ability to efficiently use the receptor molecules (ACE2 for human and civet) seems to be a major limiting factor for animal-to-human and human-to-human transmission (*35*). This also explains why the SARS-CoV was able to cause the human pandemic but the closely related bat SARS-like–CoVs were not. For the SARS-like–CoVs to infect humans, substantial genetic changes in the S1 receptor-binding domain will be necessary. These changes may be achieved in 1 of 2 possible ways. They could be achieved by genetic recombination, as coronaviruses are known to be able to recombine. For example, bat SARS-like–CoVs and another yet unknown coronavirus could coinfect an intermediate host, and the bat viruses would gain the ACE2 binding site in the S1 domain by recombination. The alternative is continuous evolution independent of recombination. Coronaviruses in bats could have a spectrum sufficiently diverse to encompass the progenitor virus for SARS-CoVs. The progenitor virus's ability to bind human ACE2 may be acquired or improved by adaptation (i.e., point mutations) in ≥1 intermediate host before it could efficiently infect humans. The existence of at least 3 discontinuous highly variable genomic regions between SARS-CoV and SARS-like–CoV indicates that the second mechanism is more likely.

In conclusion, the discovery of bat SARS-like–CoVs and the great genetic diversity of coronaviruses in bats have shed new light on the origin and transmission of SARS-CoV. Although the exact natural reservoir host for the progenitor virus of SARS-CoV is still unknown, we believe that a continued search in different bat populations in People's Republic of China and neighboring countries, combined with experimental infection of different bat species with SARS-CoV, will eventually identify the native reservoir species. A positive outcome of these investigations will greatly enhance our understanding of spillover mechanisms, which will in turn facilitate development and implementation of effective prevention strategies. The discovery of SARS-like–CoVs in bats highlights the increasingly recognized importance of bats as reservoirs of emerging viruses (*36*). Moreover, the recent emergence of SARS-CoVs and other bat-associated viruses such as henipaviruses (*37*,*38*), Menangle, and Tioman viruses (*36*), and variants of rabies viruses and bat lyssaviruses (*38*,*39*) also supports the contention that viruses, especially RNA viruses, possess more risk than other pathogens for disease emergence in human and domestic mammals because of their higher mutation rates (*40*).

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References

1. Peiris JSM, Guan Y, Yuen KY. Severe acute respiratory syndrome. Nat Med. 2004;10:S88–97.
2. Liang G, Chen QX, Xu JG, Liu YF, Lim W, Peiris JSM, et al. Laboratory diagnosis of four recent sporadic cases of community-acquired SARS, Guangdong Province, China. Emerg Infect Dis. 2004;10:1774–81.
3. Song HD, Tu CC, Zhang GW, Wang SY, Zheng K, Lei LC, et al. Cross-host evolution of severe acute respiratory syndrome coronavirus in palm civet and human. Proc Natl Acad Sci U S A. 2005;102:2430–5.
4. Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science. 2003;300:1394–9.
5. Marra MA, Jones SJ, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YS, et al. The genome sequence of the SARS-associated coronavirus. Science. 2003;300:1399–404.
6. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med. 2003;348:1953–66.
7. Xu RH, He JF, Evans MR, Peng GW, Field HE, Yu DW, et al. Epidemiologic clues to SARS origin in China. Emerg Infect Dis. 2004;10:1030–7.
8. Centers for Disease Control and Prevention. Prevalence of IgG antibody to SARS-associated coronavirus in animal traders—Guangdong Province, China, 2003. MMWR Morb Mortal Wkly Rep. 2003; 52:986–7.

9. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in Southern China. Science. 2003;302:276–9.
10. Chinese SARS Molecular Epidemiology Consortium. Molecular evolution of the SARS coronavirus during the course of the SARS epidemic in China. Science. 2004;303:1666–9.
11. Wu D, Tu C, Xin C, Xuan H, Meng Q, Liu Y, et al. Civets are equally susceptible to experimental infection by two different severe acute respiratory syndrome coronavirus isolates. J Virol. 2005;79:2620–5.
12. Fouchier RA, Kuiken T, Schutten M, Van Amerongen G, van Doornum GJ, van den Hoogen BG, et al. Aetiology: Koch's postulates fulfilled for SARS virus. Nature. 2003;423:240.
13. Qin C, Wang JW, Wei Q, She MP, Marasco WA, Jiang H, et al. An animal model of SARS produced by infection of *Macaca mulatta* with SARS coronavirus. J Pathol. 2005;206:251–9.
14. Li BJ, Tang Q, Cheng D, Qin C, Xie FY, Wei Q, et al. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. Nat Med. 2005;11:944–51.
15. Bukreyev A, Lamirande EW, Buchholz UJ, Vogel LN, Elkins WR, St Claire M, et al. Mucosal immunisation of African green monkeys (*Cercopithecus aethiops*) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. Lancet. 2004;363:2122–7.
16. Martina BE, Haagmans BL, Kuiken T, Fouchier RA, Rimmelzwaan GF, van Amerongen G, et al. Virology: SARS virus infection of cats and ferrets. Nature. 2003;425:915.
17. Roberts A, Vogel L, Guarner J, Hayes N, Murphy B, Zaki S, et al. Severe acute respiratory syndrome coronavirus infection of golden Syrian hamsters. J Virol. 2005;79:503–11.
18. Liang L, He C, Lei M, Li S, Hao Y, Zhu H, et al. Pathology of guinea pigs experimentally infected with a novel reovirus and coronavirus isolated from SARS patients. DNA Cell Biol. 2005;24:485–90.
19. Roberts A, Paddock C, Vogel L, Butter E, Zaki S, Subbarao K. Aged BALB/c mice as a model for increased severity of severe acute respiratory syndrome in elderly humans. J Virol. 2005;79:5833–8.
20. Subbarao K, Mcauliffe J, Vogel L, Fahle G, Fischer S, Tatti K, et al. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. J Virol. 2004;78:3572–7.
21. Chen W, Yan M, Yang L, Ding B, He B, Wang YZ, et al. SARS-associated coronavirus transmitted from human to pig. Emerg Infect Dis. 2005;11:446–8.
22. Weingartl HM, Copps J, Drebot MA, Marszal P, Smith G, Gren J, et al. Susceptibility of pigs and chickens to SARS coronavirus. Emerg Infect Dis. 2004;10:179–84.
23. Ng SKC. Possible role of an animal vector in the SARS outbreak at Amoy Gardens. Lancet. 2003;362:570–2.
24. Swayne DE, Suarez DL, Spackman E, Tumpey TM, Beck JR, Erdman D, et al. Domestic poultry and SARS coronavirus, southern China. Emerg Infect Dis. 2004;10:914–6.
25. Wang M, Yan M, Xu H, Liang W, Kan B, Zheng B, et al. SARS-CoV infection in a restaurant from palm civet. Emerg Infect Dis. 2005;11:1860–5.
26. Tu C, Crameri G, Kong X, Chen J, Sun Y, Yu M, et al. Antibodies to SARS coronavirus in civets. Emerg Infect Dis. 2004;10:2244–8.
27. Kan B, Wang M, Jing H, Xu H, Jiang X, Yan M, et al. Molecular evolution analysis and geographic investigation of severe acute respiratory syndrome coronavirus-like virus in palm civets at an animal market and on farms. J Virol. 2005;79:11892–900.
28. Poon LLM, Chu DKW, Chan KH, Wong OK, Ellis TM, Leung YHC, et al. Identification of a novel coronavirus in bats. J Virol. 2005;79:2001–9.
29. Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeek H, Dobson AP. The ecology of wildlife diseases. Oxford (UK): Oxford University Press; 2002.
30. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. Science. 2005;310:676–9.
31. Lau SKP, Woo PCY, Li KSM, Huang Y, Tsoi HW, Wong BHL, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc Natl Acad Sci U S A. 2005;102:14040–5.
32. Woo PC, Lau SK, Li KS, Poon RW, Wong BH, Tsoi HW, et al. Molecular diversity of coronaviruses in bats. Virology. 2006;351:180–7.
33. Tang XC, Zhang JX, Zhang SY, Wang P, Fan XH, Li LF, et al. Prevalence and genetic diversity of coronaviruses in bats from China. J Virol. 2006;80:7481–90.
34. Childs JE. Zoonotic viruses of wildlife: hither from yon. Arch Virol Suppl. 2004;18:1–11.
35. Li W, Wong S-K, Li F, Kuhn JH, Huang I-C, Choe H, et al. Animal origins of the severe acute respiratory syndrome coronavirus: insight from ACE2-S-protein interactions. J Virol. 2006;80:4211–9.
36. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T. Bats: important reservoir hosts of emerging viruses. Clin Microbiol Rev. 2006;19:531–45.
37. Breed AC, Field HE, Epstein JH, Daszak P. Emerging henipaviruses and flying foxes–conservation and management perspectives. Biological Conservation. 2006;131:211–20.
38. Mackenzie JS, Field HE. Emerging encephalitogenic viruses: lyssaviruses and henipaviruses transmitted by frugivorous bats. Arch Virol Suppl. 2004;18:97–111.
39. Harris SL, Brookes SM, Jones G, Huston AM, Racey PA, Aegerter J, et al. European bat lyssaviruses: distribution, prevalence and implications for conservation. Biological Conservation. 2006;131:193–210.
40. Cleaveland S, Laurenson MK, Taylor LH. Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philos Trans R Soc Lond B Biol Sci. 2001; 356:991–9

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Risk Factors for Human Infection with Avian Influenza A H5N1, Vietnam, 2004

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To evaluate risk factors for human infection with influenza A subtype H5N1, we performed a matched case-control study in Vietnam. We enrolled 28 case-patients who had laboratory-confirmed H5N1 infection during 2004 and 106 age-, sex-, and location-matched control-respondents. Data were analyzed by matched-pair analysis and multivariate conditional logistic regression. Factors that were independently associated with H5N1 infection were preparing sick or dead poultry for consumption ≤7 days before illness onset (matched odds ratio [OR] 8.99, 95% confidence interval [CI] 0.98–81.99, p = 0.05), having sick or dead poultry in the household ≤7 days before illness onset (matched OR 4.94, 95% CI 1.21–20.20, p = 0.03), and lack of an indoor water source (matched OR 6.46, 95% CI 1.20–34.81, p = 0.03). Factors not significantly associated with infection were raising healthy poultry, preparing healthy poultry for consumption, and exposure to persons with an acute respiratory illness.

The first indication that the current epizootic of highly pathogenic avian influenza subtype H5N1 (influenza A H5N1) would have a serious effect on human heath occurred in early 2004, when influenza H5N1 was identified in a series of patients admitted to the National Pediatric Hospital in Hanoi with severe viral pneumonia (*1*). Since then, large-scale and global spread of the disease in poultry has been accompanied by sporadic cases in humans. Despite many millions of avian infections and >200 human cases, knowledge of influenza H5N1 remains inadequate. Neither how these viruses are transmitted to humans nor, consequently, the most effective way to reduce the risk for infection is fully understood. Descriptive and analytic epidemiologic studies conducted in Hong Kong Special Administrative Region, People's Republic of China, during the 1997 outbreak of influenza H5N1 (*2–5*) identified visiting a live bird market as a risk factor. However, the current outbreak encompasses different viruses and different sociodemographic, farming, and behavioral contexts. Several seroprevalence studies of healthcare workers and a case-control study from Thailand have been published from the current outbreak (*6–9*), but further work is needed to develop and test hypotheses on the mechanism of transmission of influenza H5N1 to humans. To clarify the source and mode(s) of transmission of influenza H5N1 to humans and to guide the control and prevention of influenza, we conducted a case-control study of all cases of avian influenza H5N1 identified in humans in Vietnam in 2004.

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Materials and Methods

All persons with laboratory-confirmed influenza A H5 cases detected in Vietnam from January 1 through December 31, 2004, were eligible for enrollment as case-patients. Case-patients were identified from persons hospitalized with an acute respiratory infection considered by clinicians, on the basis of clinical and epidemiologic findings, to have a suspected case of H5N1 infection. Clinicians did not use a systematic case definition or screening protocol to identify patients eligible for testing for H5N1 infection. Throat swabs or tracheal aspirate samples were sent to the National Institute of Hygiene and Epidemiology in Hanoi or to the Pasteur Institute in Ho Chi Minh City for reverse transcription (RT) PCR and viral isolation. When possible, samples with positive results for influenza A H5 were sent to a World Health Organization (WHO) reference laboratory for confirmatory diagnosis.

For each case-patient, 4 control-respondents, individually matched by gender, age (age difference ≤24 months), and place of residence (same ward or village), were selected by use of a random number table from a list of persons fitting the selection criteria provided by the community health station near each case-patient's place of residence. Potential control-respondents were excluded if they reported having suffered an illness with respiratory symptoms and fever (temperature ≥38°C) during their matched case-patient's period of illness (onset to recovery or death). If the selected control-respondent refused to participate or did not meet the inclusion criteria, the geographically closest eligible person was then selected from the list. All eligible control-respondents were asked to provide a throat swab and venous blood (5 mL) to confirm that they were not currently or had not previously been infected with influenza A H5. Participation of case-patients and control-respondents was voluntary and required written consent and, for those aged <18 years, signature of a parent or guardian.

Trained interviewers administered a structured questionnaire to case-patients and control-respondents. If the case-patient or control-respondent was a child or if the case-patient had died, the questionnaire was administered to a proxy, usually the child's parent or a close family member living in the same household. The questionnaire collected information about demographic characteristics; preexisting health status; smoking behavior; potential animal, human, and environmental exposures to influenza A H5; and personal and household hygienic practices. Case-patients or their proxies were asked about exposures in the 7 days before illness onset, and control-respondents were asked about exposures during the same 7-day period as their matched case-patient.

Definitions

Persons who met any of the following criteria were considered to be laboratory-confirmed influenza A H5 case-patients: 1) influenza A H5–specific RNA detected in a single specimen by RT-PCR by using 2 different primer pairs; 2) influenza A H5 detected in a single specimen by RT-PCR identification and by sequencing or virus isolation; 3) influenza A H5–specific RNA detected by RT-PCR in 2 different specimen types (e.g., throat swab and tracheal aspirate); and 4) influenza A H5–specific RNA detected by RT-PCR in 2 samples taken on different days. Control-respondents were considered to be true control-respondents if throat swab specimens were negative for influenza A H5–specific RNA by RT-PCR and anti-H5 antibodies could not be detected in serum samples by microneutralization assay (*10*).

Laboratory Methods

Influenza A H5 subtype–specific RNA was detected in clinical samples by RT-PCR with primers that targeted regions of the hemagglutinin gene of the influenza H5N1 virus developed by WHO, the US Centers for Disease Control and Prevention (CDC), and the Government Virus Unit in Hong Kong. Clinical specimens were injected into Madin-Darby canine kidney cells for virus isolation, and RT-PCR was used to identify influenza A H5. Specimens and cell cultures suspected of containing influenza A H5 were handled according to recognized biosafety standards.

Serum samples were immediately processed, stored at –25°C, and shipped frozen on dry ice to CDC. To measure influenza A H5–specific antibody, microneutralization assay was conducted as previously described (*10*) by using H5N1 viruses A/Vietnam/1194/2004 and A/Vietnam/3212/2004. Microneutralization test results were considered to be positive if an anti-H5 titer of >40 was obtained by 2 independent assays.

Statistical Analysis

Data entry and analysis of individual explanatory variables was performed by using Epi-Info 6 (CDC, Atlanta, GA, USA). Mantel-Haenszel matched-pair analysis (McNemar test) was used to estimate the strength and statistical significance of associations between exposures and influenza A H5 infection. An association was considered statistically significant if 2-sided tests of significance had a p-value ≤0.05. To examine independence of effects, multivariate conditional logistic regression was performed by using the conditional logistical regression (CLogit) function in Stata/SE 8.0 for Windows (Stata Corp LP, College Station, TX, USA). Any variables with p≤0.2 after matched analysis were included in the initial model. A backward stepwise variable- selection strategy was used to construct a final model with a significance level of >0.1 for removal and a significance level of <0.05 for re-entry into the model. Persons missing data for variables under study were excluded from any analysis involving the missing variable. Collinearity was assessed by generating a correlation coefficient matrix for all variables to be considered for inclusion in the regression model. The presence of effect-measure modification by age and sex was assessed for all variables in the final model by entering product terms. A final model was achieved by entering the variables retained in the backward selection model.

The attributable risk percent (AR%) was estimated as follows: ([odds ratio (OR) – 1]/OR) ×100. The population attributable risk percent (PAR%) was estimated as follows: PAR% = AR% × proportion of case-patients exposed.

Results

A total of 28 laboratory-confirmed influenza A H5 cases were detected in 2004 from 15 provinces of Vietnam; 21 (75%) were fatal. All 28 cases were RT-PCR positive

for influenza A subtype H5 at either the National Institute of Hygiene and Epidemiology, Hanoi, or the Pasteur Institute, Ho Chi Minh City; H5N1 virus was isolated in 12. The diagnosis of influenza A H5 infection was independently confirmed for 25: 20 at a WHO reference laboratory and 5 at the Oxford University Clinical Research Unit in Vietnam.

The interviews began on February 7, 2004, at which time 20 of the 28 case-patients had already been identified. The interval between onset of illness and interview was a mean of 35.7 days; the maximum interval was 63 days. The mean age of case-patients was 14 years (range 1–31 years, median 15 years), and 9 (32%) patients were children <10 years of age. The numbers of male and female case-patients were equal (14 each). Among confirmed case-patients were 2 family clusters (mother and daughter and 2 sisters). A total of 106 control-respondents were enrolled, 4 per case-patient, except for 3 case-patients aged <5 years for whom only 1, 2, and 3 control-respondents could be recruited per case, respectively. All control-respondents were negative for avian influenza A H5–specific RNA by RT-PCR and for anti-H5 antibodies by microneutralization assay. None of the case-patients or control-respondents worked in the commercial (industrial) poultry-raising sector.

The results of matched-pair analysis are shown in Table 1. Direct handling of sick or dead poultry in the 7 days before onset of illness had the strongest point estimate of effect (matched OR 31) and high statistical significance ($p<0.001$) despite wide confidence limits (95% confidence interval [CI] 3.4–1150). The presence of sick or dying poultry in the household (matched OR 7.4, 95% CI 2.7–59) or neighborhood (matched OR 3.9, 95% CI 1.0–55.7) was also statistically associated with infection as was the absence of an indoor water source in the household (matched OR 5.0, 95% CI 1.3–77.0) and education to high school level or higher (matched OR 16.0, 95% CI 1.2–594.1).

Eight variables with $p\leq0.2$ were considered for inclusion in the conditional logistic regression model to estimate independence of effects. Although significantly associated with infection in the single-variable analysis, the presence of sick or dead poultry in the neighborhood was excluded from the final regression model because missing data for this variable led to the exclusion of 36 participants (6 case-patients and 30 control-respondents). Educational level was excluded because it was not a relevant variable for the 13 case-patients <15 years of age. Because of the 2 family clusters, each comprising 2 case-patients, the influence of clustering of household-level factors on the regression model was investigated by running the regression model first with all cases and then again including only 1 case from each of these 2 households. All 4 variations of 1 case from each household were run. Because the outcomes of these different approaches did not differ, all cases were included in the final model.

The final conditional logistic regression model included 3 variables as independent risk factors for H5N1 infection (Table 2). Of the 28 case-patients, 16 (57%) had either sick or dead poultry in their household or had directly prepared sick or dead poultry for consumption; another 6 reported sick or dead poultry in the neighborhood. Of the 28 case-patients, 22 (79%) did not have an indoor water source. No statistically significant effect-measure modification was detected.

Among persons who prepared sick or dead poultry for consumption, the proportion of H5N1 cases attributable to this practice (AR%) is estimated in this study to be 89% ($[(8.99 - 1)/8.99] \times 100$). However, because only 32% of all case-patients reported this practice, stopping this practice would prevent only an estimated 28% of H5N1 cases ($PAR\% = 0.89 \times 0.32$).

Discussion

Source of Infection

This study identified the presence in the household and the handling of dead or sick poultry in an H5N1-affected area as risk factors for human H5N1 infection. Although not surprising, these findings reinforce the hypothesis that close contact with infected domestic poultry is the primary source of transmission of influenza H5N1 to humans. The absence of a statistical association between infection and contact with other animals such as pigs, cats, or dogs is reassuring. Replication and excretion of H5N1 by asymptomatic domestic waterfowl has been demonstrated and is a plausible source of infection for humans (*11*); however, although a 1997 case-control study found that visiting live poultry markets was a risk factor for human influenza A H5N1 infection (*2*), our study and the study from Thailand (*9*) did not identify contact with healthy poultry as a risk factor. However, viral titers in asymptomatic waterfowl may be much lower than in diseased poultry (*11*) and may therefore pose a low risk to humans, which our study was underpowered to identify.

Despite evidence that limited human-to-human transmission of H5N1 has occurred (*3*,*5*,*12*), we observed no significant differences between case-patients and control-respondents in terms of exposure to persons who might be a source of H5N1 infection, e.g., patients with an acute respiratory infection. This finding is consistent with that of the case-control study in Thailand (*9*).

Route of Transmission

Our study found an association between infection and direct and household contact with diseased poultry. This

Table 1. Matched-pair analysis of potential risk factors for human infection with avian influenza A H5N1, Vietnam, 2004

Exposure and characteristics	Case-patients (n = 28), n (%)	Control-respondents (n = 106), n (%)	Matched OR* (95% CI)	p value
High school, college, or university education (persons >14 y of age)	8 (53)†	17 (29)‡	16.0 (1.2–594.1)	0.03
Family size >5 persons	8 (29)	32 (30)	1.2 (0.4–4.0)	0.88
Ever smoked	3 (11)	10 (9)	2.0 (0.1–30.5)	0.91
Chronic medical conditions	3 (11)	9 (8)	1.3 (0.2–7.7)	0.93
Poultry-related exposures§				
Prepared and cooked healthy poultry	9 (32)	24 (23)	2.2 (0.6–10.4)	0.249
Prepared and cooked sick or dead poultry	9 (32)	6 (6)	31.0 (3.4–1150)	<0.001
Helped prepare or cook sick or dead poultry	7 (25)	12 (11)	2.6 (0.8–8.7)	0.102
Bought live poultry for household consumption	3 (11)	9 (8)	1.2 (0.2–7.0)	0.895
Bought freshly killed poultry for household consumption	0 (0)	11 (10)	Incalculable	-
Live poultry in household	18 (64)	52 (49)	3.0 (0.9–10.0)	0.103
Sick or dead poultry in household	15 (54)	20 (19)	7.4 (2.7–59.0)	<0.001
Live poultry in neighborhood	19 (79)¶	75 (74)#	1.07 (0.2–6.6)	0.810
Sick or dead poultry in neighborhood	12 (43)	29 (27)	3.9 (1.0–55.7)	0.05
Farm or family with >150 poultry within 100 m	4 (14)	16 (15)	1.0 (0.2–4.2)	0.742
Household members work with commercial poultry	1 (4)	2 (2)	2.0 (0.0–38.4)	0.88
Other animal-related exposures				
Pigs in household	9 (32)	28 (26)	1.4 (0.3–6.4)	0.838
Pig in neighborhood	15 (54)	48 (45)	2.0 (0.5–7.2)	0.505
Dogs in household	18 (64)	58 (55)	1.7 (0.6–4.7)	0.430
Cats in household	9 (32)	23 (22)	2.0 (0.6–5.9)	0.374
Buffalo in household	1 (4)	1 (1)	4.0 (0.1–314)	0.86
Cows in household	5 (18)	14 (13)	2.4 (0.3–17.4)	0.581
Human-related exposures§				
Exposed to patients with acute respiratory infection (temperature ≥38°C)	6 (21)	11 (10)	2.4 (0.7–13.5)	0.145
Exposed to hospitalized patients with acute respiratory infection	5 (18)	9 (8)	2.4 (0.6–12.9)	0.210
Hygiene- and environment-related exposures				
Handwashing before eating (usually or sometimes)	23 (82)	90 (85)	1.3 (0.3–5.6)	0.911
Handwashing >3 times/d	25 (89)	87 (82)	0.53 (0.1–2.4)	0.568
Wading in ponds, rice fields, ditches	4 (14)	7 (7)	2.4 (0.4–19.2)	0.469
No indoor water source in household	22 (79)	64 (60)	5.0 (1.3–77.0)	0.024
Poor hygiene conditions**	12 (43)	47 (44)	1.0 (0.3–3.4)	0.829

*Matched analysis using McNemar (Mantel-Haenszel) test statistics; 95% confidence limits are exact intervals for maximum likelihood estimate; OR, odds ratio; CI, confidence interval.
†n = 15.
‡n = 58.
§7 d before illness onset in case-patient.
¶n = 24.
#n = 102.
**A composite measure of 7 indicators: dust level in person's home, type of flooring, frequency of house cleaning, habit of washing hands before eating, habit of washing fruit before eating, estimated frequency of handwashing/d, and interviewer's assessment of household cleanliness.

association, if true, could operate by 2 mechanisms. First, transmission may be by inhalation or conjunctival deposition of large infectious droplets, which may travel only short distances (*13*); second, the presence of infected poultry in the home and preparation of infected poultry for consumption may result in exposure to higher virus concentrations than other types of exposure. An alternative hypothesis is that the consumption, rather than the preparation, of infected poultry is the route of infection. Domestic cats and possibly tigers have been infected by the oral route (*14*,*15*), and the observed association between preparation and infection could be the result of a confounding association between preparing an infected bird (apparent risk factor) and consuming an infected bird (true risk factor). Unfortunately, the study did not ask about consumption of sick or dead poultry because the gastrointestinal route of transmission was not considered plausible at the time the study was designed.

The unexpected finding that the absence of an indoor water source is associated with infection may point toward a role for self-inoculation into conjunctival, nasal, or oral mucosa by contaminated hands or possibly foodstuffs. Certainly, the environmental stability of avian influenza viruses is sufficient for this to occur (*16*), and hand-

Table 2. Results of multivariate analysis of potential risk factors for human infection with avian influenza A H5N1, Vietnam, 2004*

Exposure and characteristics	Odds ratio	95% CI	p value
Prepare and cook sick or dead poultry	8.99	0.98–81.99	0.052
Sick or dead poultry in household	4.94	1.21–20.20	0.026
No indoor water source in household	6.46	1.20–34.81	0.03

*Conditional logistic regression; final model with 3 variables entered; χ^2 for likelihood ratio test = 28.35; p<0.001; no. observations = 134; CI, confidence interval.

washing has been shown to decrease risk for respiratory infections (*17*). However, we asked 2 questions specifically about handwashing behavior, and neither was significantly associated with infection (Table 1). An alternative, and more controversial, explanation is that people without access to an indoor water source may acquire infection by drinking or washing in outdoor water sources contaminated with feces from infected poultry. This hypothesis is plausible, given the extended survival of avian influenza viruses in water (*18,19*) and the demonstration of oral infectivity in cats (*14*). Although an indoor water source might simply be a proxy indicator of socioeconomic status or the priority the household gives to hygiene, all other hygiene factors and a composite hygiene index did not show a statistically significant association with infection.

That 5 case-patients did not report any exposure to sick poultry in the 7 days before illness onset has several possible explanations: recall bias by case-patients or their proxies, infection acquired from infected but asymptomatic animals such as ducks, an incubation period >7 days, or infection from a contaminated environment as discussed above.

Study Limitations

A major source of potential bias in this and other studies of risk factors for human H5N1 infection is the use of self-reported prior exposure to sick poultry as a screening tool for identifying potential case-patients. Use of this tool introduces a selection bias that favors finding greater exposure to sick poultry among case-patients than other groups, regardless whether the relationship is causal. In our study, clinicians were not using a systematic screening tool to identify possible H5N1 case-patients, but knowledge of poultry as the source of human H5N1 infection was ubiquitous. Possibly, H5N1 case-patients who did not have exposure to sick poultry may have been less likely to be identified than case-patients who did report this exposure. However, 5 of the 28 case-patients (18%) did not report exposure to sick poultry, indicating that exposure to sick poultry was not a prerequisite for identification as a case-patient. In light of this conflict between clinical necessity and study purity, estimates of the size of the association between exposure to sick poultry and H5N1 infection could be interpreted as maximums that are likely to have been inflated by this selection bias.

The relatively small number of case-patients means that the study may be underpowered to detect factors posing only a moderate risk for infection and to detect effect modification. A standardized questionnaire and trained interviewing staff were used to try to minimize interviewer bias, but masking the interviewers as to the case or control status of the respondents was not possible. Recall bias was likely to have occurred, especially because the high case-fatality rate meant that a larger proportion of interviews in the case group (26/28) than the control group (35/106) were completed by proxies. The substantial delay between onset of illness and interviews (mean 35.7 days) is also a potential source of recall bias.

The finding of a significant positive association between level of education and risk for infection was unexpected and is difficult to explain. It may be the consequence of a bias introduced by proxy respondents for deceased case-patients reporting higher levels of education than case-patients had actually achieved.

Misclassification of case-patients and control-respondents was unlikely. All control-respondents were demonstrated to have no detectable antibodies to H5N1, and all case-patients had a clinically compatible illness with laboratory evidence of H5N1 infection, which was independently verified for 25 (89%) of the 28 cases.

Public Health and Research Implications

Preparing sick or dead poultry for consumption in an H5N1-affected area is a risky practice. Although this study cannot estimate the absolute risk, among those who prepared sick or dead poultry for consumption, a high proportion of infections could be attributed to this practice. However, as the practice was not that widespread in our study participants, stopping it would prevent only an estimated 28% of H5N1 cases. Less risky but more widespread practices probably account for a greater proportion of H5N1 cases; these practices must also be identified and tackled. Regardless whether consumption of infected poultry is itself a risk factor, preparation and consumption of sick or dead poultry in infected areas must stop. That all 106 persons selected as control-respondents from communities with at least 1 confirmed human H5N1 case were negative for H5N1 antibodies adds further evidence to the belief that widespread subclinical H5N1 infection has not yet occurred in Southeast Asia (*20*).

The finding of an association between lack of access to an indoor water source and H5N1 infection provides an interesting basis for formulating new hypotheses, but it is not sufficiently strong evidence for concluding that H5N1 transmission is occurring by water or as a result of inadequate hygiene. Despite 2 reports of exposure to potentially contaminated water in Vietnamese H5N1 case-patients ([*21*]; pers. comm., Ministry of Health, Vietnam), no human cases of H5N1 infection have been directly attributed to exposure to contaminated water. Nevertheless, hygiene practices and access to safe water have collateral benefits regardless of H5N1 and should be encouraged and pursued. Environmental investigations are needed to sample water sources in and around the households of incident H5N1 case-patients and compare the findings to water sources sampled in and around unaffected households.

Familial clusters of cases have been a significant feature of the epidemiology of H5N1 infection since 2004 in that numerous clusters have occurred in Vietnam, Thailand, Cambodia, Indonesia, and Turkey (*22*,*23*). Although common exposures and behavior may be one explanation for the marked clustering, most clusters have involved blood relatives such as sibling pairs or parent-child groups rather than unrelated pairs such as husbands and wives. This finding suggests that inherited biologic factors, such as sialic acid receptor phenotype or immune response, may be determinants of infection and disease. Studying intrinsic determinants of susceptibility will require pooling of data and samples from affected families across affected countries. If intrinsic susceptibility were a risk determinant across affected countries, it might dilute associations between certain behavior and infection unless the analyses were undertaken within subgroups that are homogeneous with respect to their intrinsic susceptibility. In this respect, intrafamilial studies that combine measures of biologic susceptibility with data about behavioral patterns, including food consumption and hygiene practices, may be particularly enlightening.

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References

1. World Health Organization. Avian influenza A (H5N1) in humans and poultry in Viet Nam, 2004 January 13 [cited 2006 May 18]. Available from http://www.who.int/csr/don/2004_01_13/en/index.html
2. Mounts AW, Kwong H, Izurieta HS, Ho Y, Au T, Lee M, et al. Case-control study of risk factors for avian influenza A (H5N1) disease, Hong Kong, 1997. J Infect Dis. 1999;180:505–8.
3. Buxton Bridges C, Katz JM, Seto WH, Chan PK, Tsang D, Ho W, et al. Risk of influenza A (H5N1) infection among health care workers exposed to patients with influenza A (H5N1), Hong Kong. J Infect Dis. 2000;181:344–8.
4. Bridges CB, Lim W, Hu-Primmer J, Sims L, Fukuda K, Mak KH, et al. Risk of influenza A (H5N1) infection among poultry workers, Hong Kong, 1997–1998. J Infect Dis. 2002;185:1005–10.
5. Katz JM, Lim W, Bridges CB, Rowe T, Hu-Primmer J, Lu X, et al. Antibody response in individuals infected with avian influenza A (H5N1) viruses and detection of anti-H5 antibody among household and social contacts. J Infect Dis. 1999;180:1763–70.

6. Liem NT, Lim W; World Health Organization International Avian Influenza Investigation Team, Vietnam. Lack of H5N1 avian influenza transmission to hospital employees, Hanoi, 2004. Emerg Infect Dis. 2005;11:210–5.
7. Apisarnthanarak A, Erb S, Stephenson I, Katz JM, Chittaganpitch M, Sangkitporn S, et al. Seroprevalence of anti-H5 antibody among Thai health care workers after exposure to Avian influenza (H5N1) in a tertiary care center. Clin Infect Dis. 2005;40:e16–8.
8. Schultsz C, Dong VC, Chau NV, Le NT, Lim W, Thanh TT, et al. Avian influenza H5N1 and healthcare workers. Emerg Infect Dis. 2005;11:1158–9.
9. Centers for Disease Control and Prevention. Investigation of avian influenza (H5N1) outbreak in humans—Thailand, 2004. MMWR Morb Mortal Wkly Rep. 2006;55(Suppl 1):3–6.
10. Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W, et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol. 1999;37:937–43.
11. Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, et al. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol. 2005;79:11269–79.
12. Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med. 2005;352:333–40.
13. Bridges CB, Kuehnert MJ, Hall CB. Transmission of influenza: implications for control in health care settings. Clin Infect Dis. 2003;37:1094–101.
14. Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA, et al. Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol. 2006;168:176–83.
15. Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, et al. Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis. 2004;10:2189–91.
16. Shortridge KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, et al. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology. 1998;252:331–42.
17. Luby SP, Agboatwalla M, Feikin DR, Painter J, Billhimer W, Altaf A, et al. Effect of handwashing on child health: a randomised controlled trial. Lancet. 2005;366:225–33.
18. Ito T, Okazaki K, Kawaoka Y, Takada A, Webster RG, Kida H. Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch Virol. 1995;140:1163–72.
19. Stallknecht DE, Shane SM, Kearney MT, Zwank PJ. Persistence of avian influenza viruses in water. Avian Dis. 1990;34:406–11.
20. Vong S, Coghlan B, Mardy S, Holl D, Seng H, Ly S, et al. Low frequency of poultry-to-human H5N1 virus transmission, southern Cambodia, 2005. Emerg Infect Dis. 2006;12:1542–7.
21. de Jong MD, Bach VC, Phan TQ, Vo MH, Tran TT, Nguyen BH, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med. 2005;352:686–91.
22. World Health Organization. Situation updates–avian Influenza [cited 2006 May 18]. Available from http://www.who.int/csr/disease/avian_influenza/updates/en/index.html
23. Olsen SJ, Ungchusak K, Sovann L, Uyeki TM, Dowell SF, Cox NJ, et al. Family clustering of avian influenza A (H5N1). Emerg Infect Dis. 2005;11:1799–801.

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Salmonella Enteritidis in Broiler Chickens, United States, 2000–2005

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US Department of Agriculture Food Safety and Inspection Service (FSIS) data on *Salmonella enterica* serotype Enteritidis in broiler chicken carcass rinses collected from 2000 through 2005 showed the annual number of isolates increased >4-fold and the proportion of establishments with *Salmonella* Enteritidis–positive rinses increased nearly 3-fold (test for trend, p<0.0001). The number of states with *Salmonella* Enteritidis in broiler rinses increased from 14 to 24. The predominant phage types (PT) were PT 13 and PT 8, 2 strains that a recent Foodborne Diseases Active Surveillance Network (FoodNet) case-control study associated with eating chicken. FSIS is directing more sampling resources toward plants with marginal *Salmonella* control to reduce prevalence in products including broilers. The policy targets establishments with common *Salmonella* serotypes of human illness, including *Salmonella* Enteritidis. Voluntary interventions should be implemented by industry.

During the 1990s, *Salmonella enterica* serotype Enteritidis briefly surpassed *S.* Typhimurium as the predominant *Salmonella* serotype isolated from humans in the United States (*1*). Eggs were frequently implicated as the cause of outbreaks of human infection (*2*,*3*), and the outbreak strain was often detected in the implicated egg production flock (*4*). After egg producers implemented quality assurance programs in the late 1990s, human *Salmonella* Enteritidis infection rates decreased by ≈50% (*1*).

Recently, 2 US case-control studies in Foodborne Diseases Active Surveillance Network (FoodNet) sites identified eating chicken as a risk factor for sporadic human *Salmonella* Enteritidis infection (*5*,*6*), replicating findings of a case-control study performed in England in the late 1980s (*7*). While the overall incidence of human salmonellosis in FoodNet sites was lower in 2005 than in the mid-1990s, the incidence of *Salmonella* Enteritidis infections was ≈25% higher (*8*). We present US Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) *Salmonella* testing program data collected from 2000 to 2005 that suggest a need for interventions to prevent the emergence of this *Salmonella* serotype in broiler chickens in the United States.

Methods

FSIS *Salmonella* Testing Program

As of January 2000, an FSIS performance standard for *Salmonella* was set for all establishments that slaughter US broiler chickens (*9*). Establishments that slaughtered >20,000 chickens per year were eligible for FSIS regulatory *Salmonella* testing. These establishments accounted for >95% of raw poultry marketed in the United States.

The sampling frame for the present study included all eligible FSIS-inspected establishments. Each month, eligible facilities were randomly selected for *Salmonella* testing to begin in the following month. In each broiler slaughter setting that was tested, 1 broiler chicken carcass rinse (hereafter referred to as broiler rinse) was collected per day for 51 days of operation. The 51 broiler rinses constitute a "*Salmonella* set." Sets were scheduled approximately once a year. When a plant did not meet the *Salmonella* performance standard, a follow-up set was scheduled. To limit bias, this report does not include data from follow-up sets.

Carcasses were collected after they exited the chiller, downstream from the slaughter line. The chiller is designed to bring carcass temperatures down to the refrigeration range. The postchill collection site was selected as

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the sampling site because interventions for pathogen reduction are generally located before this point.

Broiler Rinse Collection

Carcasses were collected after they exited the chiller and aseptically placed in a sterile bag. A 400-mL volume of buffered peptone water was added to the carcass in the bag. Half the volume was poured into the interior cavity and the other half over the skin. The carcass was rinsed with a rocking motion for 1 minute at a rate of ≈35 cycles per minute. After the carcass was removed from the bag, the rinse was poured into a sterile container and shipped on a freezer pack by overnight mail to 1 of 3 FSIS laboratories (Athens, GA; Alameda, CA; St. Louis, MO, USA) for analysis (*10*).

Microbiologic Testing

Testing of broiler rinses for *Salmonella* was performed by using standard FSIS isolation methods (*11*). Before October 2003, an immunoassay system (Assurance polyclonal enzyme immunoassay, BioControl Systems, Inc., Bellevue, WA, USA) was used to screen enrichment broths for *Salmonella*. Beginning in October 2003, *Salmonella* gene amplification (BAX System PCR Assay, DuPont Qualicon, Wilmington, DE, USA) was performed on lysed cells after overnight incubation in buffered peptone broth (35°C). Broiler rinses that tested positive on the screening test were cultured for *Salmonella* with standard methods (i.e., selective enrichment, plating, serologic and biochemical confirmation). Three presumptive *Salmonella* colonies with the predominant colony form were selected from each plate for biochemical and serologic confirmation. One confirmed *Salmonella* isolate was sent to the National Veterinary Services Laboratories (NVSL, USDA-APHIS-VS, Ames, IA, USA) for *Salmonella* serotyping (*12*).

Beginning in 2001, isolates of *Salmonella* Enteritidis were phage typed at NVSL (*13*). Because the predominant *Salmonella* Enteritidis phage types were clonal (*6*,*14*) and pulsed-field gel electrophoresis and antimicrobial susceptibility patterns were not available on all isolates during the study period, no further characterization of the isolates was performed for this report.

Analysis

Analysis was restricted to *Salmonella* sets performed in calendar years 2000–2005. A χ^2 test (2-sided) was used to test trends for annual percent of *Salmonella* Enteritidis isolates among *Salmonella*-positive broiler rinses and all analyzed broiler rinses, respectively. A χ^2 test for trend was also performed to assess the percent of establishments tested annually with *Salmonella* Enteritidis–positive broiler rinses, with subanalyses by establishment size. Approximately two thirds of establishments were large (≥500 employees), one fourth were small (<500 but ≥10 employees), and 5% were very small (<10 employees). In addition, a χ^2 test for trend was performed on the number of isolates per *Salmonella* Enteritidis–positive establishment, by year (SAS version 9.1, SAS Institute, Inc., Cary, NC, USA).

The number of positive broiler rinses per state was plotted on a US map that showed the geographic density of broiler chicken production by county for the year 2002 (*15*). Results were plotted for 2 periods: calendar years 2000–2002 and 2003–2005. Phage types of isolates were tabulated by year.

The present study preceded a new FSIS policy to control *Salmonella*. The new policy emphasizes improvement in *Salmonella* control in product classes that have not reduced *Salmonella* prevalence in the past decade, such as broilers, and focuses on plants that test positive for common serotypes of human illness, such as *Salmonella* Enteriditis (*16*).

Results

During the 6-year study period, 280 (0.5%) *Salmonella* Enteritidis isolates were recovered from 51,327 broiler rinses (Table 1). From 2000 to 2005, the proportion of *Salmonella* isolates that were *Salmonella* Enteritidis increased (test for trend, p<0.0001). The percentage of all broiler rinses that tested positive also increased (test for trend, p<0.0001).

Overall, 90 establishments tested positive from 2000 through 2005. The number of establishments testing positive increased from 17 (9%) of 197 in 2000 to 47 (25%) of 187 in 2005 (test for trend, p<0.0001, Table 2). The

Table 1. *Salmonella* Enteritidis (SE) isolates per year as a proportion of *Salmonella*-positive broiler rinses and total broiler rinses, 2000–2005

Year	No. SE isolates	*Salmonella*-positive rinses	SE as a proportion of all salmonellae (%)*	No. rinses tested	SE-positive rinse as a proportion of all rinses*
2000	23	914	2.5	10,057	0.2
2001	17	1,065	1.6	8,955	0.2
2002	33	1,059	3.1	9,183	0.4
2003	29	828	3.5	6,468	0.5
2004	58	957	6.1	7,072	0.8
2005	120	1,559	7.7	9,592	1.3
Total	280	6,382	4.4	51,327	0.5

*Test for trend, p<0.0001; SE isolates as a proportion of *Salmonella* isolates and all broiler rinses by year.

Table 2. Establishments with *Salmonella* Enteritidis (SE)–positive broiler rinses, by establishment size,* 2000–2005

Year	All*		Large†		Small‡	
	No. tested	SE positive (%)	No. tested	SE positive (%)	No. tested	SE positive (%)
2000	197	17 (9)	128	9 (7)	60	8 (13)
2001	186	15 (8)	111	10 (9)	61	5 (8)
2002	185	22 (12)	123	17 (14)	53	5 (9)
2003	143	25 (17)	103	17 (17)	37	8 (22)
2004	160	25 (16)	111	16 (14)	43	9 (21)
2005	187	47 (25)	126	32 (25)	48	15 (31)

*Establishment size: large ≥500; small ≥10 to <500; very small <10 employees. (SE was not isolated in the 54 sets from very small establishments.) Ninety establishments had SE-positive broiler rinses; 63 (70%) were large and 27 (30%) were small.
†Test for trend, p<0.0001.
‡Test for trend, p<0.01.

increase in the number of positive establishments per year remained significant after stratification by large versus small establishment size. While most establishments with *Salmonella* Enteritidis–positive broiler rinses were large, in most years, a higher proportion of the small establishments that were tested had positive rinses.

During the 6-year study period, the proportion of *Salmonella* Enteritidis–positive establishments with multiple positive broiler rinses per year also increased significantly (p<0.01, test for trend, Figure 1). In addition, the proportion of establishments with ≥4 positive broiler rinses per year (of 51 broiler rinse tests per set) increased, beginning in 2002.

From 2000 to 2002, *Salmonella* Enteritidis was isolated from broiler rinses in 14 states, compared with 24 states from 2003 to 2005 (Figure 2). Phage type (PT) 13 was predominant, accounting for half of all isolates, followed by *Salmonella* Enteritidis PT 8, which accounted for more than one third of isolates (Table 3). In 2005, the number of isolates that were PT 8 increased >3-fold compared with 2004.

Discussion

The principal finding of this study was a significant increase in the number of broiler chicken slaughter establishments with *Salmonella* Enteritidis–positive broiler rinses in the years from 2000 through 2005. The 90 slaughter establishments with positive rinses were dispersed across 24 states, reflecting the geographic distribution of the US broiler industry. During the study period, increases were seen in the proportion of both large and small establishments that had such positive broiler rinses.

Some caution is warranted when interpreting our findings. The purpose of the FSIS *Salmonella* program is to assess performance of individual establishments. The program is not designed to estimate national prevalence of poultry contamination because it does not fully account for production volume or regional or seasonal effects. Furthermore, samples are collected after slaughter processes that are intended to reduce carcass contamination. Nonetheless, the apparent emergence of *Salmonella* Enteritidis in broilers is noteworthy given the increase in human *Salmonella* Enteritidis infection rates in the United States (*8*) and recent findings that eating chicken is a new and important risk factor for sporadic infection (*5*,*6*). Additional epidemiologic studies are recommended to further elucidate the role of contaminated chicken in human *Salmonella* Enteritidis infections and estimate the extent of illness attributable to chicken. Retail food surveillance and laboratory subtyping studies (*6*) may also be valuable because they enable comparisons of human and poultry strains.

In this report, 2 *Salmonella* Enteritidis phage types, PT 8 and PT 13, accounted for most isolates from broiler rinses. In a recent FoodNet study, the association between *Salmonella* Enteritidis infection and eating chicken strengthened in analyses restricted to patients infected with these 2 phage types (*6*). The possible emergence of these phage types in broiler chickens suggests that industry should implement appropriate *Salmonella* Enteritidis controls for broiler chickens (*17*,*18*).

The present study preceded a new FSIS policy to control *Salmonella* in broilers that emphasizes common serotypes of human illness (*16*). As part of this effort, FSIS

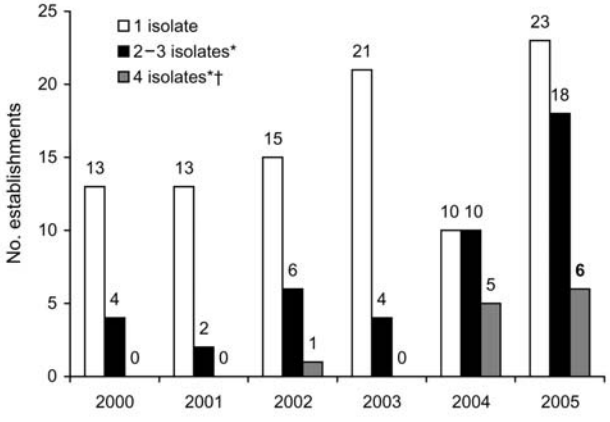

Figure 1. Number of *Salmonella* Enteritidis (SE)–positive broiler rinses, by establishment, 2000-2005. *p<0.01, test for trend. †4 establishments had 2 broiler sets in 2005; the mean number of SE isolates per set in 2005 is presented for these establishments.

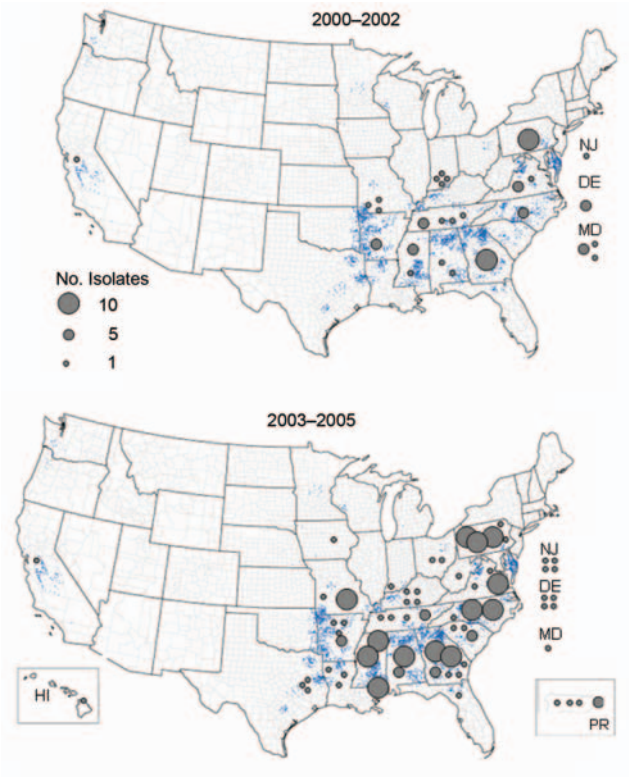

Figure 2. Geographic distribution of *Salmonella* Enteritidis isolates in broiler rinses in the first and second half of the study period (2000–2002 vs. 2003–2005). Each blue dot represents 2 million broilers produced in 2002. Broiler production data: US Department of Agriculture National Agricultural Statistics Service.

held 2 public meetings on *Salmonella* in broilers: 1 in Athens, Georgia, in August 2005 on controls before slaughter (preharvest), and another in Atlanta, Georgia, in February 2006 on controls in the slaughter plant (postharvest). Information from these meeting was used to prepare guidelines to help broiler plants control salmonellae (*19*). The agency is also monitoring progress of meat and poultry plants in controlling this organism. If, in July 2007, most plants (e.g., 90%) that manufacture a specific product (e.g., broiler carcasses) have not reduced the percentage of *Salmonella* tests that are positive to at least half the FSIS performance standard, the agency will consider actions to improve control of salmonellae. One option that FSIS is considering is to post *Salmonella* results on the web for product classes that have not made sufficient progress, listing data by plant name.

In the 1990s, successful voluntary quality assurance programs to control *Salmonella* Enteritidis were developed by the egg industry and state poultry health officials (*20*). Many of the interventions are adaptable to the control of this organism in broilers. For example, control points for the organism in broilers are likely to include monitoring and sanitation of breeding flocks, hatcheries, broiler flocks, and slaughter establishments. Serotype data that FSIS provides to plants on each isolate as part of its new *Salmonella* policy (*16*) may also assist plant officials to make informed SE risk management decisions.

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Dr Altekruse is a veterinary epidemiologist in the Public Health Service assigned to the USDA Food Safety and Inspection Service. His research interests include characterization of *Salmonella* isolates from meat and poultry and reductions in indicator and pathogen counts during slaughter.

References

1. Patrick ME, Adcock PM, Gomez TM, Altekruse SF, Holland BH, Tauxe RV, et al. *Salmonella* Enteritidis infections, United States, 1985–1999. Emerg Infect Dis. 2004;10:1–7.
2. St Louis ME, Morse DL, Potter ME, DeMelfi TM, Guzewich JJ, Tauxe RV, et al. The emergence of grade A eggs as a major source of *Salmonella* Enteritidis infections. New implications for the control of salmonellosis. JAMA. 1988;259:2103–7.
3. Trepka MJ, Archer JR, Altekruse SF, Proctor ME, Davis JP. An increase in sporadic and outbreak- associated *Salmonella* Enteritidis infections in Wisconsin: the role of eggs. J Infect Dis. 1999; 180:1214–9.
4. Altekruse S, Koehler J, Hickman-Brenner F, Tauxe RV, Ferris K. A comparison of *Salmonella* Enteritidis phage types from egg-associated outbreaks and implicated laying flocks. Epidemiol Infect. 1993;110:17–22.

Table 3. *Salmonella* Enteritidis (SE) phage types from broiler rinses, 2001–2005*

Year	SE isolates no.	Phage type			
		PT 13 no. (%)	PT 8 no. (%)	Other† no. (%)	No data‡ no. (%)
2001	17	11 (65)	4 (24)	1 (6)	1 (6)
2002	33	12 (36)	8 (24)	5 (15)	8 (24)
2003	29	13 (45)	14 (48)	1 (3)	1 (3)
2004	58	37 (64)	15 (26)	4 (7)	2 (3)
2005	120	56 (47)	50 (42)	9 (8)	5 (4)
Total	257	129 (50)	91 (35)	20 (8)	17 (7)

*Phage type (PT) data were not available for 2000. Row percents do not sum to 100 because of rounding.
†Other phage types: 13a (7); PT 23 (7); PT 28 (3); PT 2 (2); PT 14B (1).
‡No phage type data were available for 17 isolates.

5. Kimura AC, Reddy V, Marcus R, Cieslak PR, Mohle-Boetani JC, Kassenborg HD, et al. Chicken consumption is a newly identified risk factor for sporadic *Salmonella enterica* serotype Enteritidis infections in the United States: a case-control study in FoodNet sites. Clin Infect Dis. 2004;38(Suppl 3):S244–52.
6. Marcus R, Varma JK, Medus C, Boothe EJ, Anderson BJ, Crume T, et al. Re-assessment of risk factors for sporadic *Salmonella* serotype Enteritidis infections: a case-control study in five FoodNet sites, 2002–2003. Epidemiol Infect. 2006;7:1–9.
7. Cowden JM, Lynch D, Joseph CA, O'Mahony M, Mawer SL, Rowe B, et al. Case-control study of infections with *Salmonella* Enteritidis phage type 4 in England. BMJ. 1989;299:771–3.
8. Centers for Disease Control and Prevention. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food—10 states, United States, 2005. MMWR Morb Mortal Wkly Rep. 2006;55:392–5.
9. Food Safety and Inspection Service. Pathogen reduction; hazard analysis and critical control point (HACCP) systems; Final Rule. Fed Regist. 1996;61:38805–55 [cited 2006 Oct 11]. Available from http://www.fsis.usda.gov/OPPDE/rdad/FRPubs/93-016F.htm
10. Food Safety and Inspection Service. FSIS directive 10,230.5: Self-instruction guide for collecting raw meat and poultry product samples for *Salmonella* analysis. February 4, 1998. [cited 2006 Oct 11]. Available from http://www.fsis.usda.gov/OPPDE/rdad/FSISDirectives/10230-5.pdf
11. Food Safety and Inspection Service. Microbiology laboratory guidebook. August 17, 2006 [cited 2006 Oct 11]. Available from http://www.fsis.usda.gov/Science/Microbiological_Lab_Guidebook
12. Ewing E, editor. Edwards and Ewing's identification of *Enterobacteriaceae*. 5th ed. London: Elsevier; 1986.
13. Ward LR, de Sa JD, Rowe B. A phage-typing scheme for *Salmonella* Enteritidis. Epidemiol Infect. 1987;99:291–4.
14. Laconcha I, Baggesen DL, Rementeria A, Garaizar J. Genotypic characterisation by PFGE of *Salmonella enterica* serotype Enteritidis phage types 1, 4, 6, and 8 isolated from animal and human sources in three European countries. Vet Microbiol. 2000;75:155–65.
15. USDA National Agricultural Statistics Service. Number of broilers and other meat-type chickens sold: 2002 [cited 2006 Oct 11]. Available from http://www.nass.usda.gov/research/atlas02/Livestock/Poultry/Number%20of%20Broilers%20and%20Other%20Meat-Type%20Chickens%20Sold.gif
16. Food Safety and Inspection Service. *Salmonella* verification sample result reporting: agency policy and use in public health protection. Fed Regist. 2006;71:9772–7.
17. Gast RK, Holt PS. Experimental horizontal transmission of *Salmonella* Enteritidis strains (phage types 4, 8, and 13a) in chicks. Avian Dis. 1999;43:774–8.
18. McIlroy SG, McCracken RM, Neill SD, O'Brien JJ. Control, prevention and eradication of *Salmonella* Enteritidis infection in broiler and broiler breeder flocks. Vet Rec. 1989;125:545-8.
19. Food Safety and Inspection Service. Compliance guideline for controlling *Salmonella* in poultry. 1st ed. August 2006 [cited 2006 Oct 11]. Available from http://www.fsis.usda.gov/PDF/Compliance_Guideline_Controlling_Salmonella_Poultry.pdf#search=%22Compliance%20guideline%20for%20controlling%20Salmonella%20in%20poultry%22
20. Mumma GA, Griffin PM, Meltzer MI, Braden CR, Tauxe RV. Egg quality assurance programs and egg-associated *Salmonella* Enteritidis infections, United States. Emerg Infect Dis. 2004;10:1782–9.

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Human Metapneumovirus in Turkey Poults

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This study was conducted to reexamine the hypothesis that human metapneumovirus (hMPV) will not infect turkeys. Six groups of 2-week-old turkeys (20 per group) were inoculated oculonasally with 1 of the following: noninfected cell suspension; hMPV genotype A1, A2, B1, or B2; or avian metapneumovirus (aMPV) subtype C. Poults inoculated with hMPV showed nasal discharge days 4–9 postexposure. Specific viral RNA and antigen were detected by reverse-transcription PCR and immunohistochemical evaluation, respectively, in nasal turbinates of birds exposed to hMPV. Nasal turbinates of hMPV-infected turkeys showed inflammatory changes and mucus accumulation. Each of the 4 hMPV genotypes caused a transient infection in turkeys as evidenced by clinical signs, detection of hMPV in turbinates, and histopathologic examination. Detailed investigation of cross-species pathogenicity of hMPV and aMPV and its importance for human and animal health is needed.

Human metapneumovirus (hMPV) is a negative-sense, single-stranded, nonsegmented RNA virus of the genus *Metapneumovirus* of subfamily *Pneumovirinae* of the family *Paramyxoviridae*, a large family of viruses that affect humans and animals (*1*). Recently identified, hMPV is noted for causing respiratory tract infections in children (*2*). The virus was isolated from nasopharyngeal swabs collected from 28 children over a period of 20 years in the Netherlands, and it is thought to have been circulating in human populations for at least 50 years (*2*). hMPV has 2 major genetic lineages (A and B) and at least 4 subgroups identified so far, according to analysis of fusion, attachment glycoprotein, and phosphoprotein genes (*3*). The virus has worldwide distribution, and reports have identified hMPV from the United States, Canada, United Kingdom, Italy, Germany, France, Israel, Australia, Asian countries, and Peru (*4*,*5*).

The disease caused by hMPV ranges from mild upper respiratory tract infection to severe bronchiolitis or bronchitis and pneumonia. It affects all age groups, but it is more severe in young, elderly, and immunocompromised persons (*6*). Serologic surveys indicate that the virus is ubiquitous in nature and that new infections can occur throughout life because of incomplete protection and genetic heterogeneity of the virus (*3*,*7*,*8*). Clinically, the disease is similar to that of human respiratory syncytial virus and is second only to human respiratory syncytial virus as 1 of the leading causes of bronchiolitis in young children (*9*).

Recent studies have shown that avian metapneumovirus (aMPV) subtype C isolates from domestic turkeys and wild birds in the United States show high sequence homology to hMPV (*10*,*11*). Both viruses belong to genus *Metapneumovirus* and share a projected amino acid identity of 56%–88% (*11*). An upper respiratory tract pathogen of poultry, aMPV has global distribution (*12*). The United States was considered free of aMPV until an outbreak of respiratory tract infection occurred in turkey flocks in Colorado in May 1996 (*13*). Later, in 1997, the disease appeared in Minnesota, the largest turkey-producing state in the country. Serologic evidence now exists for the spread of aMPV into neighboring states of Iowa, Wisconsin, South Dakota, and North Dakota (*14*). The aMPV C identified in the United States has been reported only in North America and is antigenically and genetically different from subtypes A, B, and D detected in Europe, Asia, Africa, and South America (*15*–*18*).

Although hMPV and aMPV are closely related, they are not reported to cause cross-infection. A previous attempt to experimentally infect chickens and turkeys with hMPV

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was not successful (*2*). Our objective was to reexamine the hypothesis that hMPV will not infect turkeys by exposing 2-week-old turkeys to 4 genotypes of hMPV.

Materials and Methods

Cells and Virus

LLC-MK2 cells (ATCC no. CCL-7) were maintained in Minimum Essential Medium (MEM) (Invitrogen, Grand Island, New York, USA) supplemented with 3% bovine fetal serum, 2 mmol/L L-glutamine, nonessential amino acids, 100 IU/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. Four genotypes of hMPV based on G gene were provided by the University of Iowa for this study: A1 (GenBank accession no. DQ312456), A2 (DQ312449), B1 (DQ312452), and B2 (DQ312457). Genotype A1 was isolated from a nasal wash sample of a 3-year-old girl by propagation on LLC-MK2 cells for 29 days. Genotypes A2 and B1 were isolated from nasal wash samples of 7- and 1-year-old girls, respectively, by propagation on LLC-MK2 cells for 14 days. Genotype B2 was isolated from a nasopharyngeal sample of a 19-year-old woman by propagation on LLC-MK2 cells for 20 days. After primary isolation, these viruses were further propagated in LLC-MK2 cells in opti-MEM (Invitrogen) with 2 μg/mL trypsin (replenished every other day), 100 IU/mL of penicillin G sodium, 100 μg/mL streptomycin, and no serum.

aMPV C was isolated from the nasal turbinates of 8-week-old turkeys with acute upper respiratory tract infection. The virus was passaged 6 times on chicken embryo fibroblasts, then 6 times on Vero cells. The virus, designated as aMPV/Minnesota/Turkey/19/2003 (aMPV MN 19), was used with a titer of 10^5 50% tissue culture infective dose ($TCID_{50}$)/mL.

Birds

A total of 120 2-week-old turkey poults (Large White, Nicholas) from an aMPV-naive breeder flock that was not vaccinated for aMPV were used (Institutional Biosafety Committee [ID 785] and Institutional Animal Care and Use Committee approved protocol #0505A69986, University of Minnesota, Saint Paul, MN, USA). These turkey poults were free of aMPV, *Mycoplasma meleagridis, M. synoviae, M. gallisepticum, Bordetella avium,* and Newcastle disease virus.

Experimental Design

Turkey poults were divided into 6 groups of 20 each. Poults in each group were inoculated oculonasally with 200 μL (50 μL each in each eye and nostril) of 1 of the following: noninfected LLC-MK2 cell suspension (sham-inoculated controls), 1 of the 4 genotypes of hMPV (A1, A2, B1, B2), or aMPV C (positive controls). Poults in the 4 treatment groups received fresh, untitrated virus propagated in LLC-MK2 cells. To ascertain the amount of virus inoculated into poults in each group, each inoculum was later titrated by serial dilutions in LLC-MK2 cells. Poults were monitored daily for clinical signs. Two randomly selected poults from each group were killed for necropsy and sample collection at days 3, 5, and 7 postexposure. Nasal turbinates, tracheas, and lungs were tested for viral RNA by reverse-transcription (RT) PCR with specific primers (consensus primers for all of the 4 genotypes) for hMPV and aMPV as described below. Tissue sections were stained with hematoxylin and eosin and examined for histopathologic lesions and for viral antigen by immunohistochemical methods (*19*). Sera collected from poults in each group at days 14 and 21 postexposure were tested with aMPV-ELISA (*20*). No convincing evidence exists for any cross-reactivity between hMPV and aMPV.

Clinical Sign Scoring

Turkey poults were monitored daily for any clinical signs. A clinical sign scoring system was used to record signs shown by those exposed to the viruses (*21*). Briefly, poults in each group were given a score of 0 when they showed no clinical signs; a score of 1 for unilateral nasal discharge; 2 for bilateral nasal discharge; or 3 for thick, copious, bilateral nasal discharge. Unilateral sinus swelling was recorded as score 1, bilateral sinus swelling as 2, unilateral conjunctivitis as 1, and bilateral conjunctivitis as 2. The total score for each bird was expressed as the sum of individual scores mentioned above.

RT-PCR

Nasal turbinates, tracheas, and lungs were collected in MEM (Invitrogen) containing 100 IU/mL penicillin G sodium and 100 μg/mL streptomycin to prepare a 20% tissue homogenate. After centrifugation at 3,000 × *g* for 10 min to remove tissue debris, an additional centrifugation was performed at 8,000 × *g* for 10 min, and the supernatant was collected and used for RT-PCR. Viral RNA was extracted from the tissue homogenate supernatant by using a commercial viral RNA extraction kit (Qiagen, Valencia, CA, USA). One-step RT-PCR was performed by using a commercially available 1-step RT-PCR kit (Qiagen). Two primers designed on the basis of the hMPV fusion protein gene, 5′-GAGCAAATCCCAGACA-3′ and 5′-GAAA ACTGCCGCACAACATTTAG-3′, were used as forward and reverse primers, respectively (*22*). A 50-μL reaction was set for each tissue sample with the following temperature conditions: reverse transcription at 50°C for 30 min, initial denaturation at 94°C for 10 min, 35 cycles of annealing at 54°C for 1 min, extension at 72°C for 1 min, and denaturation at 94°C for 30 sec, followed by a final

extension at 72°C for 10 min. The RT-PCR products were then analyzed by electrophoresis on a 1.2% agarose gel. A separate RT-PCR was set for each tissue sample by using specific primers designed from the matrix protein gene of aMPV to look for any cross-reactivity. The protocol described by Shin et al. (*23*) was used. The forward primer was 5′-ACAGTGTGTGAGTTAAAAG-3′ (M1) starting from base 335, and the reverse primer was 5′- TGAC TTCAGGACATATCTC-3′ (M2) starting from base 754 of the US isolate of aMPV (aMPV/Minnesota/Turkey/2a/1997).

Virus Isolation

Nasal turbinate homogenate from poults exposed to the 4 genotypes of hMPV was injected onto LLC-MK2 cells for virus isolation. Five blind passages (14 days each) were done, and the cells were examined for any cytopathic effects. At the endpoint of each passage, 3 cycles of freeze-thaw were done and the cell culture supernatant was tested for hMPV RNA by RT-PCR as described above.

Histopathologic and Immunohistochemical Evaluations

Nasal turbinates, tracheas, and lungs were fixed in 10% buffered neutral formalin. Tissue sections were stained with hematoxylin and eosin and examined for histopathologic changes.

An immunoperoxidase procedure (*19*) originally developed to detect aMPV antigen was modified to detect hMPV antigen in formalin-fixed nasal turbinate, trachea, and lung tissues by using hMPV B2 polyclonal sera from rabbits. The tissue sections were also tested by using aMPV polyclonal sera from rabbits (*19*).

Serologic and Bacteriologic Examination

Serum samples were collected from turkey poults at days 14 and 21 postexposure. Samples were examined for antibodies by an aMPV-ELISA (*20*) that used aMPV subtype C whole-antigen–coated plates and anti-turkey IgG conjugate as the secondary antibody.

To exclude the possibility of any bacterial infections, nasal and tracheal swabs were streaked on blood agar and McConkey agar plates. The plates were incubated at 37°C for 3 days, after which they were examined for any bacterial pathogens.

Results

Virus Titers

The 4 genotypes of hMPV showed cytopathic effects in LLC-MK2 cells 10–14 days postinoculation. Cytopathic effects consisted mainly of cell rounding and formation of syncytium. hMPV genotypes A1, A2, and B1 inocula had a titer of 10^3 $TCID_{50}$/mL each. Genotype B2 inoculum had a titer of 10^5 $TCID_{50}$/mL. The aMPV C inoculum had a titer of 10^5 $TCID_{50}$/mL.

Clinical Sign Scoring

Poults inoculated with any of the 4 hMPV genotypes had unilateral or bilateral nasal discharge (Table 1; Figure 1), which varied from watery to thick mucus. Clinical signs started on day 4 postexposure and stopped on day 9 postexposure. Those infected with hMPV A1 had a clinical score range of 1–2, and 6 of 20 showed clinical signs. Poults infected with hMPV A2 had a score range of 1–8, and 12 of 20 showed clinical signs. Those inoculated with hMPV B1 had a score range of 1–7, and 7 of 20 showed clinical signs. The group inoculated with hMPV B2 had the most poults that showed clinical signs (14 of 20); score range was 2–8. Poults inoculated with aMPV MN 19 showed severe clinical signs and had an average score of 14.17 (Table 1). The main signs were thick, mucous, bilateral nasal discharge and infraorbital sinus swelling.

RT-PCR

At day 3 postexposure, RT-PCR detected hMPV viral RNA in the nasal turbinates of poults in each group exposed to hMPV. Viral RNA was also detected at day 5 postexposure in 1 bird exposed to hMPV B2 (Table 2). Tracheas and lungs did not show any viral RNA. Sham-inoculated birds were negative for hMPV. The aMPV RNA was not detected in unexposed or hMPV-exposed poults. Those infected with aMPV showed viral RNA in nasal turbinate (Table 2)

Table 1. Clinical sign scores of turkey poults exposed and not exposed to human or avian metapneumovirus*

Treatment group	Clinical sign score						Average score
	4 d (n = 18)	5 d (n = 18)	6 d (n = 16)†	7 d (n = 16)†	8 d (n = 14)‡	9 d (n = 14)‡	
Noninfected control	0	0	0	0	0	0	0
hMPV A1	2.0	2.0	2.0	2.0	1.0	0	1.5
hMPV A2	8.0	6.0	4.0	4.0	3.0	1.0	4.34
hMPV B1	1.0	1.0	4.0	7.0	3.0	0	2.66
hMPV B2	8.0	6.0	8.0	8.0	6.0	2.0	6.34
aMPV C	14.0	15.0	15.5	16.5	13.5	10.5	14.17

*d, days postexposure; hMPV, human metapneumovirus genotypes A1, A2, B1, B2; aMPV C, avian metapneumovirus subtype C.
†For the group exposed to hMPV B2, n = 15 because of 1 death (cause unknown) on day 5 postexposure.
‡For the group exposed to hMPV B2, n = 13 because of 1 death (cause unknown) on day 5 postexposure.

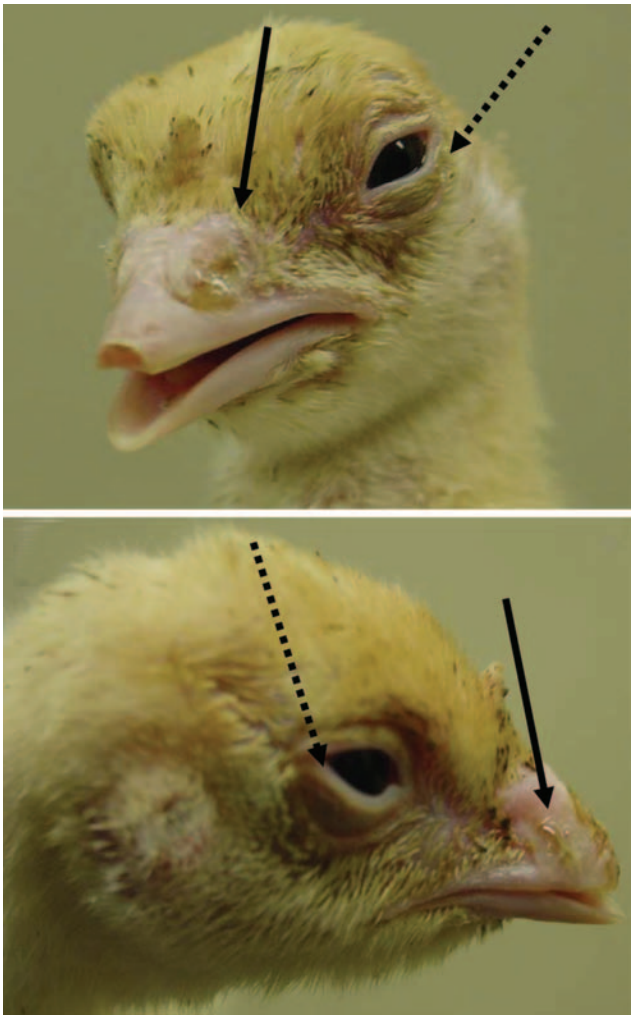

Figure 1. Clinical signs of turkey poults exposed to human metapneumovirus B2, showing nasal discharge (solid arrows) and swollen eyelids (dotted arrows).

and trachea homogenate (data not shown) by aMPV specific RT-PCR on days 3, 5, and 7 postexposure.

Virus Isolation

No cytopathic effects were detected in LLC-MK2 cell cultures inoculated with the nasal turbinate homogenate from birds exposed to any one of the 4 genotypes of hMPV. No hMPV RNA was detected from the cell culture supernatant by RT-PCR after 5 blind passages.

Histopathologic and Immunohistochemical Evaluations

At days 3 and 5 postexposure, each of the 4 genotypes had caused mild to moderate histopathologic lesions in the nasal turbinates of poults inoculated with hMPV. Inflammatory changes were more pronounced in those inoculated with hMPV B2 than with any of the other 3 genotypes. Lesions consisted of infiltration of inflammatory cells in the lamina propria, mainly lymphocytes, macrophages, and plasma cells (Figure 2A). Mucus had accumulated in the nasal cavity, and mucous glands had dilated. Tracheas of poults inoculated with hMPV B2 showed mild inflammatory changes in the form of infiltration of inflammatory cells on day 5 postexposure (Figure 2B), whereas lungs did not show any histopathologic lesions. Tissues from sham-inoculated poults also did not show any lesions (Figures 2C and D). Nasal turbinates and tracheas of poults infected with aMPV showed infiltration of inflammatory cells (Figures 2E and F), dilation of mucosal glands, and multifocal loss of cilia in turbinates (Figure 2E).

On day 3 postexposure, immunohistochemical evaluation showed hMPV antigen in the epithelial surface of nasal turbinates of poults in each group inoculated with hMPV (Figure 3A). No antigen was detected in tracheas or lungs. On days 3, 5, and 7 postexposure, those infected with aMPV showed viral antigen in turbinate (Figure 3B) and trachea tissues (data not shown). Sham-inoculated birds did not show antigen in tissues (Figure 3C).

Table 2. Reverse-transcription PCR detection of hMPV in nasal turbinates of turkey poults exposed and not exposed to human or avian metapneumovirus*

Treatment group	No. with hMPV viral RNA		
	3 d (n = 2)	5 d (n = 2)	7 d (n = 2)
Noninfected control	0	0	0
hMPV A1	1	0	0
hMPV A2	1	0	0
hMPV B1	1	0	0
hMPV B2	2	1	0
aMPV C	2	2	2

*hMPV, human metapneumovirus genotypes A1, A2, B1, B2; d, days postexposure; aMPV C, avian metapneumovirus subtype C.

Serologic and Bacteriologic Examination

No antibodies were detected by aMPV-ELISA in sera collected from poults exposed to hMPV at 14 and 21 days postexposure. However, aMPV-infected poults had positive aMPV-ELISA results at 14 and 21 days postexposure (data not shown). No cross-reactivity between aMPV and hMPV was detected by either test conducted using aMPV specific reagents, whereas their respective controls with aMPV-infected samples showed positive reactions. No pathogenic bacterial colonies were isolated from nasal or tracheal swabs collected from any of the 120 poults.

Discussion

All 4 genotypes of hMPV did infect turkeys, as evidenced by clinical signs, RT-PCR results, immunohistochemical findings, and histopathologic changes in the nasal turbinates and tracheas of exposed turkeys. These

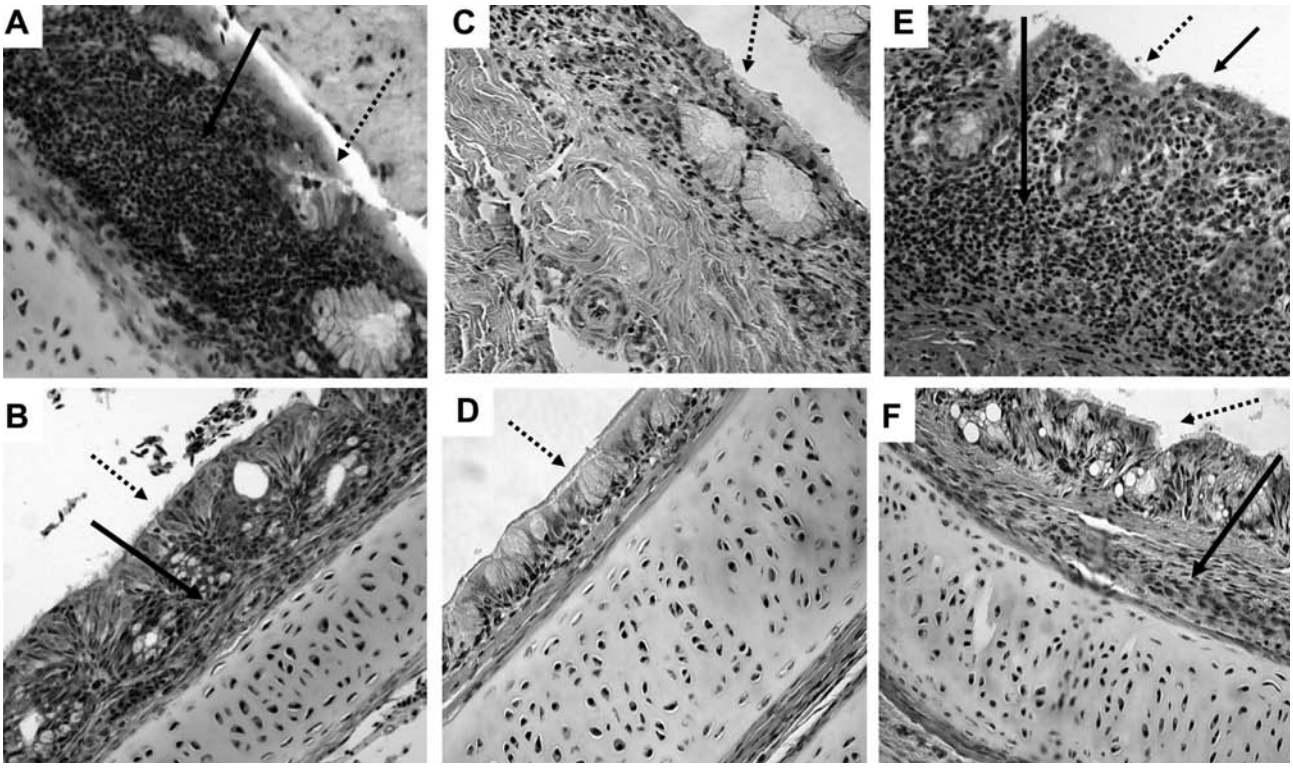

Figure 2. Histopathologic appearance of nasal turbinate and trachea tissue (magnification ×200). A) Nasal turbinates of turkey poults exposed to human metapneumovirus (hMPV) B2, showing infiltration of inflammatory cells (hematoxylin and eosin staining; solid arrow). B) Trachea of turkey poults exposed to hMPV B2, showing mild inflammation with infiltration of a few inflammatory cells in the lamina propria (solid arrow). C) Nasal turbinate of sham-inoculated turkey poults. D) Trachea of sham-inoculated turkey poults. E) Nasal turbinate of turkey poults exposed to avian metapneumovirus (aMPV C), showing infiltration of inflammatory cells and multifocal loss of cilia (solid arrows). F) Trachea of turkey poults exposed to aMPV C, showing mild inflammation with infiltration of a few inflammatory cells in the lamina propria (solid arrow). Dotted arrows indicate mucosal surface.

findings contradict those of van den Hoogen et al., who detected neither clinical signs nor virus replication in juvenile turkeys inoculated with hMPV nor experimental reproduction of infection in chickens (*2*).

In our study, 2-week-old turkeys in the four groups were inoculated oculonasally with hMPV. Those receiving either dose of hMPV (2×10^2 $TCID_{50}$ or 2×10^4 $TCID_{50}$) developed clinical signs. In contrast, van den Hoogen et al. inoculated 5×10^4 $TCID_{50}$ of hMPV in the conjunctivae and respiratory tracts of 4 juvenile turkeys (age and breed of turkeys were not mentioned) and over a 3-week period examined the birds for clinical signs and virus replication by sampling cloacal and throat swabs. Although they inoculated more virus than we did, their birds did not show clinical signs, so the amount of virus inoculated may not be the reason for the different results. Possible reasons for the contradictory results could be origin of the virus; source, age, and immune status of the turkeys used; and the samples that were analyzed. With aMPV infection, younger turkeys show more severe clinical signs than older turkeys (*19*,*21*); and nasal turbinate, a major predilection site for aMPV replication in turkeys (*21*), is where we detected hMPV.

In our study, severity of clinical signs and lesions varied with different genotypes, possibly because of differences in titers inoculated, which can potentially influence virus dissemination and pathogenicity. The virus inocula were titrated in LLC-MK2 cells after inoculating birds. The endpoint of titration was 14 days postinoculation of the virus in cell cultures; to ensure fresh inocula, we decided to use untitrated virus. The main limitation of this approach was that we could not use a uniform titer of each virus inoculum. Therefore, we could not infer any information about differences in severity of infection in turkeys with respect to different genotypes of hMPV.

Clinical signs appeared in 30%–70% birds in the groups exposed to the 4 genotypes of hMPV. The clinical signs were similar to those of birds experimentally infected with aMPV C (*21*). The main clinical sign observed in birds infected with aMPV C is watery to thick mucous discharge (*21*), and our poults exposed to hMPV showed watery to thick mucous nasal discharge. The main difference in

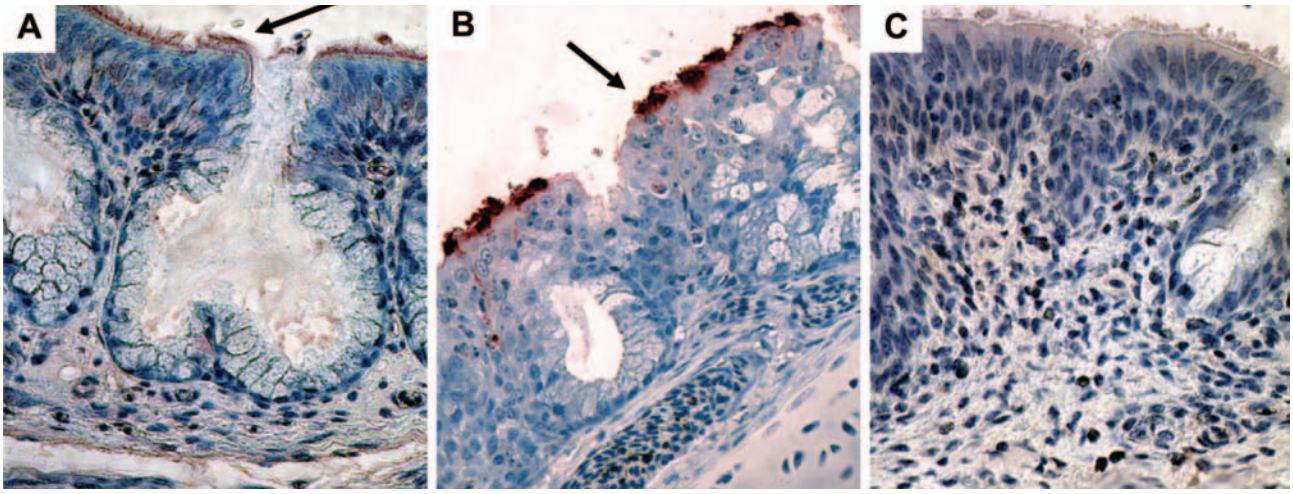

Figure 3. Immunohistochemical findings for nasal turbinates, showing peroxidase staining of viral antigen in the epithelial surface (magnification ×400; arrows). A) Turkey poults exposed to human metapneumovirus (hMPV B2). B) Turkey poults exposed to avian metapneumovirus (aMPV C). C) Sham-inoculated turkey poults.

clinical signs in our poults infected with hMPV was the absence of infraorbital sinus swelling, which is often associated with aMPV infection in turkeys (*21*).

Infiltration of inflammatory cells was observed in the nasal turbinates of poults infected with hMPV and aMPV. Nasal turbinates of poults infected with aMPV showed multifocal loss of cilia, whereas this lesion was not observed in those infected with hMPV. Immunohistochemical evaluation showed hMPV antigen in the epithelial surface of nasal turbinates; however, the staining was not as intense as we observed from aMPV infections (Figure 3B). The lack of detection of hMPV antibodies in the sera and antigens in tissues by serologic testing and immunohistochemical evaluation using aMPV reagents, respectively, indicates little to no cross-reactivity between aMPV and hMPV. However, cross-reactivity between a conserved region in aMPV nucleoprotein (N) with hMPV has been reported (*24*). We think that whole aMPV virus antigen or aMPV polyclonal serum-based detection systems may not work in the same way as affinity-purified anti-N or monoclonal antibodies.

A synthetic aMPV N-peptide–based ELISA could detect aMPV subtypes A, B, and C (*25*), whereas whole-virus–based ELISAs with plates coated with European subtypes of aMPV antigen could not detect aMPV C antibodies and vice versa (*25*). Even with aMPV subtypes, the whole-virus antigen-based detection systems did not show cross-reaction between subtypes, whereas a conserved region of nucleocapsid protein or synthetic peptide–based detection systems showed cross-reactivity. This could also occur with cross-reaction between hMPV and aMPV.

Possible zoonotic potential of metapneumoviruses and their coexistence, if any, across species barriers must be considered. Recent studies have shown that most infectious agents, especially newly emerging pathogens, can be transmitted between humans and animals (*26*). Taylor et al. reviewed literature on 1,415 species of human pathogens and identified 61% of them and 75% of emerging human disease pathogens as zoonotic. In this context, our data suggest hMPV and aMPV merit further investigations regarding cross-species transmission (*26*).

Mice, cotton rats, hamsters, ferrets, and primate species such as rhesus monkeys and African green monkeys have been used to experimentally reproduce the disease caused by hMPV (*27*,*28*). Hamsters, ferrets, and green monkeys have been shown to replicate hMPV and produce neutralizing antibodies (*27*). Replication of the virus in infected lung was also experimentally shown in mice and cotton rats (*28*). Although hMPV replicated in the upper and lower respiratory tract of hamsters and ferrets, these animals did not show any clinical signs (*27*). Hamelin et al. reported that BALB/c mice infected with hMPV showed ruffled hair, breathing difficulty, and weight loss (*28*). On the other hand, our infected turkeys showed respiratory signs in the form of watery to thick mucous nasal discharge and eyelid swelling.

Histopathologic changes in the form of interstitial inflammation and alveolitis have been observed in cotton rats and mice infected with hMPV (*28*). In our turkey poults, we did not detect inflammatory lesions in the lungs, although nasal turbinates and tracheas showed infiltration of inflammatory cells.

In our study, each of the 4 genotypes of hMPV caused a transient infection in turkey poults, as evidenced by clinical sign scores from days 4 to 9 postexposure, detection of hMPV RNA in nasal turbinates at days 3 and 5 postexposure, and histopathologic lesions in the turbinates and tracheas. Ours is the first report of an experimental infection

of turkeys with hMPV, and it opens up the possibility of using turkeys as infection models for this virus. Our data indicate a need for detailed investigation of the cross-species pathogenicity of hMPV and aMPV and significance of these viruses for human and animal health.

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References

1. Alexander DJ. Newcastle disease and other *Paramyxoviridae* infections. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM, editors. Diseases of poultry. 10th ed. Ames (IA): Iowa State University Press; 1997. pp.541–69.
2. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med. 2001;7:719–24.
3. Hamelin ME, Boivin G. Human metapneumovirus: a ubiquitous and long-standing respiratory pathogen. Pediatr Infect Dis J. 2005;24:S203–7.
4. Hamelin ME, Abed Y, Boivin G. Human metapneumovirus: a new player among respiratory viruses. Clin Infect Dis. 2004;38:983–90.
5. Gray GC, Capuano AW, Setterquist SF, Sanchez JL, Neville JS, Olson J, et al. Human metapneumovirus, Peru. Emerg Infect Dis. 2006;12:347–50.
6. Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, Cote S, et al. Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. J Infect Dis. 2002;186:1330–4.
7. Agapov E, Sumino KC, Gaudreault-Keener M, Storch GA, Holtzman MJ. Genetic variability of human metapneumovirus infection: evidence of a shift in viral genotype without a change in illness. J Infect Dis. 2006;193:396–403.
8. Boivin G, Mackay I, Sloots TP, Madhi S, Freymuth F, Wolf D, et al. Global genetic diversity of human metapneumovirus fusion gene. Emerg Infect Dis. 2004;10:1154–7.
9. Boivin G, De Serres G, Cote S, Gilca R, Abed Y, Rochette L, et al. Human metapneumovirus infection in hospitalized children. Emerg Infect Dis. 2003;9:634–40.
10. Njenga MK, Lwamba HM, Seal BS. Metapneumoviruses in birds and humans. Virus Res. 2003;91:163–9.
11. van den Hoogen BG, Bestebroer TM, Osterhaus AD, Fouchier RA. Analysis of the genomic sequence of a human metapneumovirus. Virology. 2002;295:119–32.
12. Cook JK. Avian rhinotracheitis. Rev Sci Tech. 2000;19:602–13.
13. Senne DA, Edson RK, Pederson JC, Panigrahy B. Avian pneumovirus update. In: Proceedings of the 134th Annual Meeting of the American Veterinary Medical Association; Reno, Nevada; 1997 Jul 21–24. Schaumburg (IL): The Association; 1997. p.190.
14. Bennett RS, Nezworski J, Velayudhan BT, Nagaraja KV, Zeman DH, Dyer N, et al. Evidence of avian pneumovirus spread beyond Minnesota among wild and domestic birds in central North America. Avian Dis. 2004;48:902–8.
15. Seal BS. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. Virus Res. 1998;58:45–52.
16. Shin HJ, Cameron KT, Jacobs JA, Turpin EA, Halvorson DA, Goyal SM, et al. Molecular epidemiology of subgroup C avian pneumoviruses isolated in the United States and comparison with subgroup A and B viruses. J Clin Microbiol. 2002;40:1687–93.
17. Shin HJ, Nagaraja KV, McComb B, Halvorson DA, Jirjis FF, Shaw DP, et al. Isolation of avian pneumovirus from mallard ducks that is genetically similar to viruses isolated from neighboring commercial turkeys. Virus Res. 2002;83:207–12.
18. Lwamba HC, Halvorson DA, Nagaraja KV, Turpin EA, Swayne D, Seal BS, et al. Antigenic cross-reactivity among avian pneumoviruses of subgroups A, B, and C at the matrix but not nucleocapsid proteins. Avian Dis. 2002;46:725–9.
19. Jirjis FF, Noll SL, Halvorson DA, Nagaraja KV, Shaw DP. Pathogenesis of avian pneumovirus infection in turkeys. Vet Pathol. 2002;39:300–10.
20. Chiang S, Dar AM, Goyal SM, Sheikh MA, Pedersen JC, Panigrahy B, et al. A modified enzyme-linked immunosorbent assay for the detection of avian pneumovirus antibodies. J Vet Diagn Invest. 2000;12:381–4.
21. Velayudhan BT, McComb BC, Bennett RS, Lopes VC, Shaw DP, Halvorson DA, et al. Emergence of a virulent type C avian metapneumovirus in turkeys in Minnesota. Avian Dis. 2005;49:520–6.
22. Falsey AR, Erdman D, Anderson LJ, Walsh EE. Human metapneumovirus infections in young and elderly adults. J Infect Dis. 2003;187:785–90.
23. Shin HJ, Rajashekara G, Jirjis FF, Shaw DP, Goyal SM, Halvorson DA, et al. Specific detection of avian pneumovirus (APV) US isolates by RT-PCR. Arch Virol. 2000;145:1239–46.
24. Alvarez R, Jones LP, Seal BS, Kapczynski DR, Tripp RA. Serological cross-reactivity of members of the *Metapneumovirus* genus. Virus Res. 2004;105:67–73.
25. Alvarez R, Njenga MK, Scott M, Seal BS. Development of a nucleoprotein-based enzyme-linked immunosorbent assay using a synthetic peptide antigen for detection of avian metapneumovirus antibodies in turkey sera. Clin Diagn Lab Immunol. 2004;11:245–9.
26. Taylor LH, Latham SM, Woolhouse MEJ. Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci. 2001;356:983–9.
27. MacPhail M, Schickli JH, Tang RS, Kaur J, Robinson C, Fouchier RAM, et al. Identification of small-animal and primate models for evaluation of vaccine candidates for human metapneumovirus (hMPV) and implications for hMPV vaccine design. J Gen Virol. 2004;85:1655–63.
28. Hamelin ME, Yim K, Kuhn KH, Cragin RP, Boukhvalova M, Blanco JCG, et al. Pathogenesis of human metapneumovirus lung infection in BALB/c mice and cotton rats. J Virol. 2005;79:8894–903.

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Distinct Transmission Cycles of *Leishmania tropica* in 2 Adjacent Foci, Northern Israel

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Transmission of *Leishmania tropica* was studied in 2 adjacent foci in Israel where vector populations differ. Only *Phlebotomus sergenti* was found infected with *L. tropica* in the southern focus; *P. arabicus* was the main vector in the northern focus. Rock hyraxes (*Procavia capensis*) were incriminated as reservoir hosts in both foci. *L. tropica* strains from the northern focus isolated from sand flies, cutaneous leishmaniasis cases, and rock hyraxes were antigenically similar to *L. major*, and strains from the southern focus were typically *L. tropica*. Laboratory studies showed that *P. arabicus* is a competent vector of *L. tropica*, and *P. sergenti* is essentially refractory to *L. tropica* from the northern focus. Susceptibility of *P. arabicus* may be mediated by O glycoproteins on the luminal surface of its midgut. The 2 foci differ with respect to parasites and vectors, but increasing peridomestic rock hyrax populations are probably responsible for emergence of cutaneous leishmaniasis in both foci.

Leishmaniases are parasitic diseases with a wide range of clinical symptoms and currently threaten 350 million persons in 88 countries (*1*). In Israel and its vicinity, *Leishmania major* and *L. tropica* cause cutaneous leishmaniasis (CL), and *L. infantum* can result in visceral leishmaniasis (*2*). Until recently, relatively little information was available on the epidemiology of CL caused by *L. tropica* in this region. Outbreaks were not investigated, and cases were usually grouped together with CL cases caused by *L. major* (*3*). However, in recent years, new foci of CL caused by *L. tropica* are emerging in different parts of the country, such as the Galilee region of northern Israel and the Judean Desert east of Jerusalem that warrant thorough investigations (*4*,*5*). Clinically, lesions caused by *L. tropica* last longer and are more difficult to treat than those caused by *L. major* (*6*). Although *L. tropica* can be anthroponotic, foci in Israel appear to be zoonotic, with rock hyraxes (*Procavia capensis*) serving as probable reservoir hosts (*4*).

Leishmania development in sand flies is facilitated by interaction with midgut molecules of the vector. Laboratory studies showed that sand flies are composed of 2 groups. Species such as *Phlebotomus* (*Phlebotomus*) *papatasi*, the vector of *L. major* and *P.* (*Paraphlebotomus*) *sergenti*, the main vector of *L. tropica*, show specificity for *Leishmania* they transmit in nature (*7*,*8*). Conversely, species such as *Lutzomyia longipalpis*, the vector of *L. infantum* in South America, and many others are permissive and support development of several *Leishmania* spp. (*8*,*9*).

Studies performed with *L. major* and *P. papatasi* showed that attachment in the midgut is mediated by the major surface glycoconjugate of promastigotes, lipophosphoglycan (LPG), which interacts with *Pp*Galec, a galactose-binding molecule in the midgut of *P. papatasi* (*10*). However, the mechanism of attachment may be redundant, and another molecule on the promastigote flagellum may be involved (*11*).

Recently, the susceptibility of phlebotomine sand flies to *Leishmania* parasites was shown to correlate with O-linked glycoproteins in sand fly midgut (P. Volf, unpub. data). The permissive species have O-glycosylated epitopes on the luminal midgut surface, which may serve as binding sites for lectinlike components found on the surface of parasites (*12*,*13*). We compare midgut glycosylation patterns of 2 sand fly species, *P.* (*Adlerius*) *arabicus*

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and *P*. (*Paraphlebotomus*) *sergenti* that transmit *L*. *tropica* in 2 adjacent foci in the Galilee region of northern Israel.

L. *tropica* is genetically heterogeneous, and strains are readily distinguishable by antigenic, biochemical, and molecular techniques (*14–16*). We report findings of extensive studies in 2 adjacent CL foci that demonstrate conclusively that both vector species and parasite strains from the northern focus are different from those in the southern focus, a mere 10 km away (Figure 1).

Materials and Methods

Study Area

Studies were conducted in 2 adjacent foci in the Galilee region of northern Israel (Figure 1). The northern focus comprises several villages situated on generally south-facing slopes ≈5 km north of Lake Kinneret in the eastern lower Galilee of northern Israel (32°55′N, 35°36′W). The area investigated encompasses the villages of Amnun (at sea level), Karkom (100–150 m above sea level), and Korazim (150 m above sea level), which have ≈1,200 inhabitants living in ≈300 single-family houses surrounded by gardens and built on basalt rock. Many boulders from the cleared land have been piled into large heaps separating individual plots and surrounding the villages. These boulder mounds are inhabited by numerous rock hyraxes (*P*. *capensis*).

The southern focus includes the city of Tiberias (32°47′N, 35°32′W; population = 38,952). Studies were conducted in the outskirts of urban neighborhoods, where boulder mounds were inhabited by large populations of rock hyraxes. These neighborhoods are built on north- to northeast-facing slopes.

Collection, Dissection, and Identification of Sand Flies

Sand flies were trapped by using CDC light traps (John W. Hock, Gainesville, FL, USA) in September 2002 and 2004. Dead flies were stored in 70% alcohol and identified by using several keys (*17–19*). Live female flies were immobilized on ice, rinsed briefly in 96% ethanol, and dissected in 0.9% sterile saline. Guts were microscopically examined for parasites. Heads and genitalia were used for identification. Guts containing promastigotes were aseptically placed in glass vials (2.5 mL) containing blood agar made from defibrinated rabbit blood overlaid with a 1:1 mixture of RPMI 1640 medium and Schneider *Drosophila* cell culture medium supplemented with 10% fetal calf serum (Sigma, Saint Louis, MO, USA, and Gibco-BRL, Gaithersburg, MD, USA), 10,000 IU penicillin (Biotika, L'upca, Slovakia), 100 μg/mL amikacin (Bristol-Myers Squibb, Princeton, NJ, USA), and 1,500 μg/mL 5-fluorocytosine (Sigma). Some data on *Leishmania* isolates from the northern focus were obtained from our previously published results.

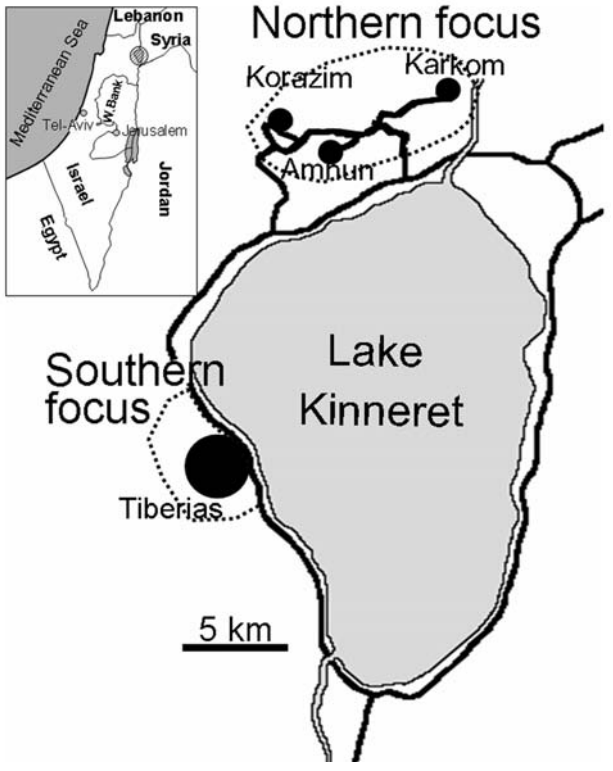

Figure 1. *Leishmania tropica* foci near Lake Kinneret in the Galilee region of Israel. Inset shows the location of the foci (circle). W. Bank, West Bank.

Collection of Animals

Rock hyraxes were trapped by using raccoon traps (http://www.havahart.com) baited with fresh leaves and anesthetized with ketamine (10 mg/kg given intramuscularly). Samples of blood and skin were obtained for parasite culture and blotted onto filter paper for PCR analysis. Animals were released at the site of capture. Skin biopsy specimens were homogenized and placed in blood agar culture medium in flat tubes (Nunclon; Nunc Nalgene International, Rochester, NY, USA). Rats (*Rattus rattus*) were trapped by using steel mesh traps (Tomahawk Live Trap Co., Tomahawk, WI, USA) placed in sewers and rock crevices. Spiny mice (*Acomys cahirinus*) were captured by using Sherman traps (H.B. Sherman Traps, Tallahassee, FL, USA). Rodents were anaesthetized with ketamine/xylazine (150 mg/kg and 15 mg/kg, respectively, given intraperitoneally). Blood from the tip of the tail was blotted on filter paper. Ear biopsy specimens were treated as described for hyraxes. Cultures were checked at 4–7-day intervals for 1 month.

DNA Extraction

DNA from wild-caught sand flies kept frozen or preserved in 100% ethanol was extracted as previously described (*20*). DNA from filter paper disks was extracted by using the phenol-chloroform method (*21*).

Detection and Identification of *Leishmania* infections by PCR

The ribosomal internal transcribed spacer region 1 (ITS1) was amplified with *Leishmania*-specific primers. ITS1 PCR products showing a *Leishmania*-specific band on agarose gels were digested with *Hae*III for species identification (*22*). Restriction fragments were subjected to electrophoresis on agarose gels and compared with DNA of *L. infantum* (Li-L699), *L. major* (Lm-L777), and *L. tropica* (Lt-L590).

Antigenic Characterization of Parasite Isolates

Initial screening of isolates was performed by using gel diffusion of glycoconjugates secreted into culture media (excreted factor) and several antileishmanial serum samples (*23*). *Leishmania*-specific monoclonal antibodies (MAbs) were used in indirect immunofluorescent antibody (IFA) assays to determine surface antigenic characteristics of parasites (*14*). Briefly, promastigotes from primary cultures of new isolates and controls of *L. infantum* (Li-L699), *L. major* (Lm-L777), and *L. tropica* (Lt-L590) were placed in wells of fluorescent antibody slides (Bellco Glass Inc., Vineland, NJ, USA), dried, and fixed in cold acetone. Slides were blocked with 5% fetal bovine serum in phosphate-buffered saline (PBS) for 1 hour at room temperature. Mouse MAbs specific for *L. major* (T1), *L. tropica* (T11, T14, and T15), *L. tropica/L. major* (T3), and *L. infantum/L. donovani* (D2) were applied for 1 hour at 37°C. Goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate was applied for 40 minutes at 37°C in the dark. The preparations were washed 3 times with PBS plus 5% Tween 20 between incubations. Slides were mounted in 3% DABCO (Sigma) in PBS/glycerol and viewed with an Axiovert microscope (Zeiss, Göttingen, Germany).

Experimental Infection of Sand Flies

Laboratory colonies of *P. sergenti* and *P. arabicus* were established from gravid females caught in the northern focus. The colonies were maintained at 23°C–25°C, 100% humidity, and 14:10 light:dark photoperiod. Adults had access to cotton wool soaked in 50% honey. Females were allowed to feed twice a week on mice anaesthetized with a ketamine/xylazine mixture (150 mg/kg and 15 mg/kg). Fed females were placed in plaster of paris–lined oviposition containers, and larvae were maintained on a decaying rabbit feces/rabbit chow mixture (*24*). Sand flies were infected by membrane feeding on heat-inactivated rabbit blood containing 5×10^5 promastigotes/mL. Fed females were maintained at 23°C and dissected on day 9 after feeding, when infections were mature. Guts were checked microscopically for *Leishmania* promastigotes. Infection intensity was scored as light (<50 promastigotes/gut), moderate (50–500 promastigotes/gut), and heavy (>500 promastigotes/gut). *L. tropica* strains from the northern (IARA/IL/2001/L810, Amnunfly1) and southern (MHOM/IL/2001/L-836, Tiberias) foci were used for comparing susceptibility of sand flies to local strains. Promastigotes from the same culture and sand flies from the same batch were used in individual experiments. For every combination, the experiment was repeated twice. Statistical tests were performed by using Statgraphics version 4.2 software (StatPoint, Englewood Cliffs, NJ, USA).

Glycosylation of Sand Fly Midguts

Midguts were dissected from 5- to 10-day-old *P. sergenti* and *P. arabicus* females. Midgut proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels under reducing conditions in a Mini-Protean III apparatus (Bio-Rad Laboratories, Hercules, CA, USA) at 200 V. Gels were stained with Coomassie brilliant blue R-250 or transferred to nitrocellulose membranes by using a Semiphor unit (Hoefer Scientific Instruments, San Francisco, CA, USA). Western blotting was performed for 90 minutes at 1.5 mA/cm^2. Membranes were incubated with 20 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween (TBS-Tw) with 5% bovine serum albumin for 2 hours and then with *Helix pomatia* agglutinin (HPA) biotinylated lectin, which recognizes N-acetyl-D-galactosamine (GalNAc), a typical carbohydrate in O-glycans. In the control groups, HPA reactions were competitively inhibited by preincubation with 250 mmol/L GalNAc for 30 minutes. After repeated washing in TBS-Tw, blots were incubated for 1 hour with streptavidin peroxidase in TBS-Tw. The peroxidase reaction was developed with the substrate 4-chloro-1-naphthol. All chemicals for lectin blotting were obtained from Sigma.

Random Amplified Polymorphic DNA Analysis

Twenty wild-caught sand flies morphologically identified as *P. sergenti*, 10 from the northern focus and 10 from the southern focus, were included in the analysis. Two flies from Tulek, Turkey, were included as an outgroup. DNA from thoraxes was extracted by using the High Pure PCR template preparation kit (Roche, Paris, France). Five decamer random primers (OPD5, OPE4, OPI1, OPI14, and OPI18; Operon Technologies Inc, Alameda, CA, USA) were used. The reaction mixture contained 12.5 μL master mixture (75 mmol/L Tris-HCl, pH 8.8, 20 mmol/L $(NH_4)_2SO_4$, 0.001% Tween 20, 800 μM deoxynucleotide

triphosphate mixture), 2.5 U *Taq* polymerase, 1.5 mmol/L $MgCl_2$, 2 μL primer (10 pmol), and 8 μL double-distilled water in a final volume of 25 μL. Random amplified polymorphic DNA (RAPD) reactions were performed in a PTC-200 thermocycler (MJ Research Inc., Waltham, MA, USA) and subjected to 45 amplification cycles. PCR products were separated by electrophoresis on a 2% agarose gel in Tris-acetate EDTA buffer at 80 V for 3 hours and stained with ethidium bromide.

ITS2 Sequencing

DNA samples for RAPD analysis were used for ITS2 sequencing. One specimen from each study area was included as previously described (*25*).

Results

Sand Fly Species

A total of 1,491 sand flies (7 species, 4 subgenera) from the northern focus and 876 sand flies (7 species, 4 subgenera) from the southern focus were identified. Phlebotomine fauna in the southern focus were relatively species poor with *P.* (*Paraphlebotomus*) *sergenti* comprising >90% of the flies. The most striking difference in the species composition between the foci was the absence of *P.* (*Adlerius*) *arabicus* and *P.* (*Adlerius*) *simici* from the southern focus, both of which were prominent species in the south-facing slopes of the northern focus (Table 1) (*26*).

Leishmania Infections in Sand Flies

To detect infections and obtain parasite isolates, sand fly females were dissected in sterile saline and guts were examined microscopically. Four (6.6%) of 61 *P. arabicus* and 1 (0.8%) of 125 *P. sergenti* from the northern focus had promastigotes in their guts. Infection intensity in *P. sergenti* from the northern focus was low, but all *P. arabicus* had heavy, mature infections. A total of 213 flies from the southern focus were dissected; 196 were *P. sergenti*, and 19 (9.7%) had promastigotes. Eleven of these females had heavy infections, and 8 had moderate-to-light infections. All infected females were caught at 1 sublocality in the southern focus, where the local infection rate was 19.6%. Promastigote cultures were established from 4 *P. arabicus* and 1 *P. sergenti* captured in the northern focus and from 18 *P. sergenti* females captured in the southern focus (Table 2). None of the other sand fly species were infected.

Sand flies that were not dissected fresh were kept frozen and were subjected to ITS1 PCR for detection of *Leishmania*. Nine (18%) of 50 *P. sergenti* females from the southern focus were positive for *Leishmania* ribosomal DNA. *Hae*III digestion of the ITS1 PCR products confirmed that all *P. sergenti* had *L. tropica* (Figure 2).

Identification of Infections in Mammals

Rodents collected in the northern focus were tested for *L. tropica* infection by ITS1 PCR. Dried blood and skin samples from 28 rats (*R. rattus*) and 46 spiny mice (*A. cahirinus)* were negative for *Leishmania* DNA. Eight of 73 rock hyraxes from the northern focus and 6 of 46 rock hyraxes from the southern focus were positive for *L. tropica* DNA by ITS-1 amplification and reverse-line blotting using sequence-specific probes (data not shown). Of the positive animals, 11 were adults (9 females and 2 males) and 1 was a juvenile male. Parasites from 1 rock hyrax captured in the northern focus were cultured and identified by ITS1 PCR and digestion with *Hae*III (Figure 3).

Antigenic Characterization of *Leishmania* Isolates

IFA assays with species-specific MAbs were used to characterize different isolates. *L. tropica* isolates from the northern focus were antigenically distinct from all other isolates, including those from the southern focus (Table 3).

Susceptibility of *P. arabicus* and *P. sergenti* to *L. tropica*

In laboratory experiments, *L. tropica* parasites from the northern focus infected only *P. arabicus*, and parasites from the southern focus infected both *P. arabicus* and *P. sergenti*. Susceptibility of *P. arabicus* for infection with *L. tropica* strains from both northern and southern foci was high (94% and 97%, respectively). In contrast, *P. sergenti* was not permissive for *L. tropica* strains from the northern focus (1 of 64 flies). Susceptibility of *P. sergenti* for

Table 1. *Phlebotomus* sand fly species in the Galilee foci, northern Israel*

	Northern focus		Southern focus	
Species	No. (%) females	No. (%) males	No. (%) females	No. (%) males
P. (*Adlerius*) *arabicus*	62 (15)	234 (22)	–	–
P. (*Adlerius*) *simici*	35 (9)	118 (11)	–	–
P. (*Paraphlebotomus*) *sergenti*	131 (32)	317 (29)	267 (91)	532 (92)
P. (*Laroussius*) *tobbi*	167 (40)	337 (31)	11 (4)	23 (4)
P. (*Laroussius*) *syriacus*	–	12 (1)	8 (3)	16 (2)
P. (*Laroussius*) *perfiliewi*	10 (2)	31 (3)	1 (<1)	2 (<1)
P. (*Phlebotomus*) *papatasi*	9 (2)	28 (3)	5 (2)	7 (1)
Total	414	1,077	292	580

*Species comprising <1% of the fauna (1 *P.* [*Paraphlebotomus*] *alexandri* and 3 *P.* [*Adlerius*] *halepensis*) found in the southern focus were not included.

Table 2. *Leishmania tropica* infection rates among *Phlebotomus* sand flies and rock hyraxes in the Galilee foci, northern Israel*

Focus	Sand flies, rate (%)		Rock hyraxes, rate (%)
	P. arabicus	*P. sergenti*	
Northern	4/61 (7)†	1/125 (1)†	8/73 (11)†
Southern	Species not found	19/196 (10)†	6/46 (13)‡

*Values are no. infected/no. tested (%). Sand fly infection rates were based on parasite isolation. Rock hyrax infection rates were determined by PCR and 1 isolate.
†*Leishmania* species confirmed by internal transcribed spacer region 1 PCR and restriction fragment length polymorphism (Figures 2 and 3).
‡*Leishmania* species confirmed by reverse line blot (data not shown).

infection with *L. tropica* from the southern focus strain was lower (66%) than that of *P. arabicus* (Figure 4).

Glycosylation of Luminal Midgut Proteins

Incubation of *P. sergenti* midgut lysates with HPA showed no reaction, indicating a lack of O-glycosylated proteins (Figure 5). In contrast, an abundant glycoprotein (37–43 kDa) was strongly labeled by HPA in *P. arabicus* midgut lysates. Controls of *P. arabicus* midgut lysates incubated with HPA blocked by preincubation with GalNAc showed no reaction, which confirmed the specificity of the lectin reactions in experimental blots (Figure 5). Labeling of midguts with fluorescein-conjugated HPA confirmed the presence of GalNAc-containing glycoproteins in the midguts of *P. arabicus*. Intensity of labeling in *P. sergenti* midguts was weaker, which reflected a nonspecific background reaction (Figure 5).

Comparison of *P. sergenti* Populations by RAPD and ITS2 Sequencing

Flies from both foci shared the same banding pattern and differed from Turkish *P. sergenti* (Figure 6). ITS 2 sequences of *P. sergenti* from both foci were identical with each other and nearly identical (99%) with the ITS 2 sequence of a *P. sergenti* specimen from the West Bank (GenBank accession no. AF462325) (data not shown).

Discussion

We have identified 2 emerging foci of CL in which rock hyraxes serve as reservoir hosts of the causative agent *L. tropica*. Despite their geographic proximity, the 2 foci show fundamental differences with regard to transmission cycles. Parasites and vector species in the southern focus are typical of most Asian zoonotic *L. tropica* foci, but the northern focus is characterized by antigenically distinct parasites that are transmitted by a newly incriminated sand fly vector.

L. tropica is widely distributed in eastern and northern Africa, the Middle East, and large parts of Asia. A recent study using 21 microsatellite loci showed that *L. tropica* is a genetically heterogeneous species composed of >80 genotypes. The genetic makeup of this complex suggests a probable African origin, with isolates from the northern focus more related to African isolates than to other strains from the Middle East (*16*).

The major surface molecule of *Leishmania* promastigotes is LPG, which has been shown to mediate attachment of parasites to the midgut of the sand fly (*8*). LPG of *L. tropica* from the northern focus is characterized by abundant terminal β-galactose residues on side chains. Conversely, β-galactose residues on LPG side chains of other *L. tropica* isolates are mostly capped with glucose (*27*). Differences in sugar moieties may have a role in infection of *P. sergenti* (Figure 4). Although β-galactose residues are present in *L. major* LPG, strains of *L. tropica* from the northern focus were not infective to *P. papatasi*, the natural vector of *L. major* (M. Svobodova, unpub. data) (*4*).

P. sergenti is probably a species complex, and its component populations show several molecular and morphologic differences (*25*). RAPD-PCR is a powerful tool for estimating genetic variability and was successfully used to compare genetic variation within and between 5 sympatric *Phlebotomus* species in Spain (*28*). Using the same primer sets, we did not find any differences between *P. sergenti* flies from the 2 foci (Figure 6). We deduce that populations from both foci are probably freely interbreeding.

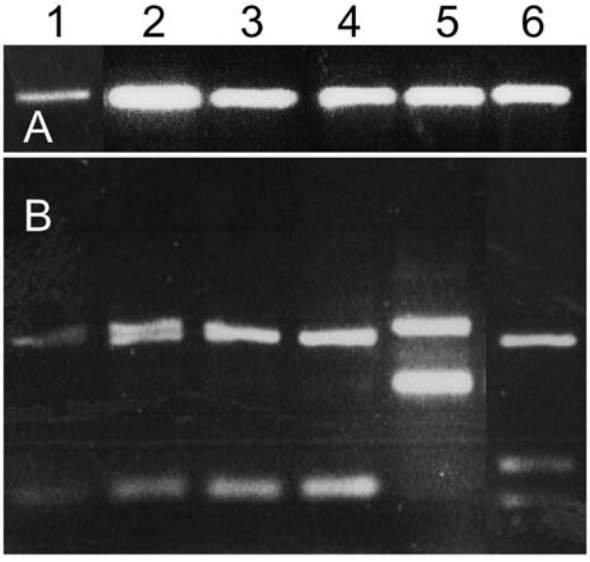

Figure 2. A) PCR of *Leishmania* internal transcribed spacer region 1 (ITS1) of naturally infected *Phlebotomus sergenti* sand flies and cultured *Leishmania* spp. controls. B) *Hae*III digestion of restriction fragment length polymorphisms of ITS1 PCR products shown in A. Lane 1, *P. sergenti* female 1; lane 2, *P. sergenti* female 2; lane 3, *P. sergenti* female 3; lane 4, *L. tropica* (Lt-L590); lane 5, *L. major* (Lm-L777); lane 6, *L. infantum* (Li-L699).

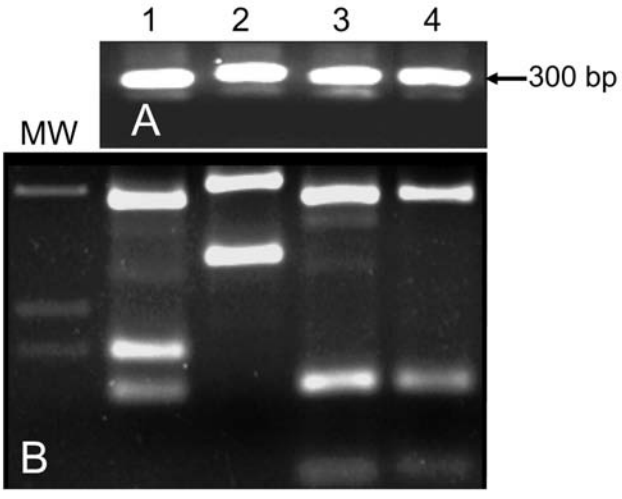

Figure 3. A) PCR of *Leishmania* internal transcribed spacer region 1 (ITS1) of cultured *Leishmania* promastigotes isolated from rock hyrax. B) *Hae*III digestion of restriction fragment length polymorphisms of ITS1 PCR products shown in A. Lane MW, molecular mass marker; lane 1, *L. infantum* (Li-L699); lane 2, *L. major* (Lm-L777); lane 3, *L. tropica* (Lt-L590); lane 4, rock hyrax.

P. sergenti is of Palaearctic origin; flies migrated into North Africa during the Miocene era (*29*). Thus, *L. tropica* and *P. sergenti* apparently originated in different continents and their geographic overlap probably arose at a later time. *P. sergenti*, *P.* (*Larroussius*) *guggisbergi*, *P.* (*Paraphlebotomus*) *saevus*, and perhaps *P. arabicus* are vectors in Africa (*30*,*31*). Since *L. tropica* variants from both foci develop in *P. arabicus*, but only the variant from the southern focus completes development in *P. sergenti*, we postulate that *L. tropica* was initially transmitted by *P. arabicus* or another permissive vector such as *P.* (*Adlerius*) *halepensis* (*9*). The more common transmission cycle is a later adaptation to *P. sergenti*, a dominant, widely distributed phlebotomine species.

Refractoriness of *P. sergenti* to variants of *L. tropica* from the northern focus is probably due to the lack of HPA-binding proteins on the luminal surface of midgut epithelium. HPA-binding epitopes are present in permissive vectors such as *P. arabicus* (Figure 4), *P. halepensis* (P. Volf, unpub. data), and *Lu. longipalpis* (*32*). These findings support infections with multiple species of *Leishmania* (*9*,*33*).

The absence of *P. arabicus* from the north-facing slopes of the southern foci contrasts dramatically with its predominance in the south-facing slopes of the northern focus. Although a satisfactory explanation for this fact is lacking, such phenomena are not unusual. For example, species richness of insects was much higher in the drier and warmer south-facing slopes of a narrow canyon (100–400 m wide) in Mount Carmel, Israel, than in the north-facing slope of the same canyon (*34*). *P. arabicus* is

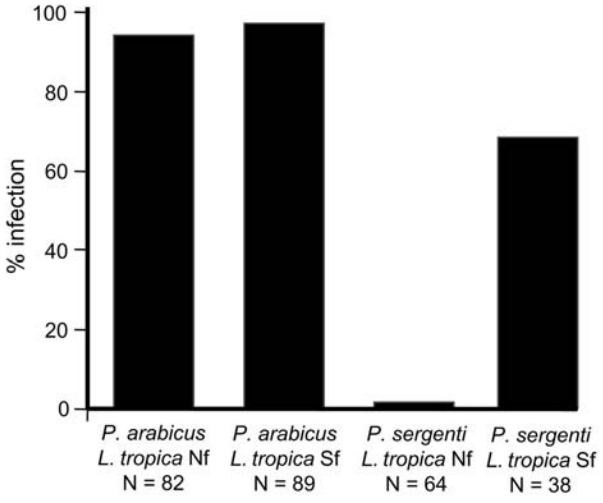

Figure 4. Artificial infection of laboratory-reared *Phlebotomus arabicus* and *P. sergenti* with *Leishmania tropica* isolates from 2 foci in Galilee, Israel. Note the high susceptibility of *P. arabicus* for both strains and refractoriness of *P. sergenti* for the northern strain. Nf, northern focus; Sf, southern focus.

Table 3. Characterization of *Leishmania tropica* isolates from the Galilee foci, northern Israel*

Focus/ source	Monoclonal antibody specificity			Excreted factor serotype
	L. major T1	*L. major*/*L. tropica* T3	*L. tropica* T11	
Northern				
Phlebotomus arabicus	5+	5+	±	A4
Girl with CL†	4+	5+	–	A4
Rock hyrax	5+	5+	±	A4
Southern				
P. sergenti	–	4+	3+	A9B2
Man with CL†	–	2+	4+	A9B2
Reference strains				
L. major	5+	5+	–	A1
L. tropica	±	3+	3+	A9

*Characterization was performed by using excreted factor serotyping (*23*) and species-specific monoclonal antibodies (*14*). CL, cutaneous leishmaniasis. Values indicate relative intensity of fluorescence under UV light. *L. tropica* isolates from the northern focus were antigenically similar to *L. major* and distinct from other *L. tropica* strains.
†Specimens were isolated by skin scraping for diagnostic purposes at the Department of Dermatology at Hadassah Hospital, Jerusalem.

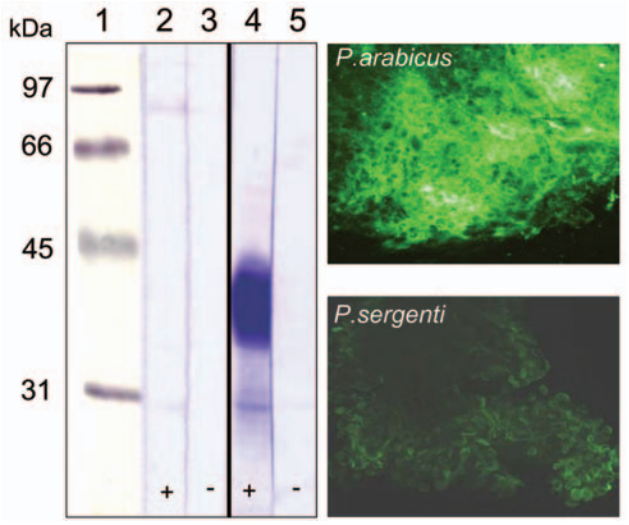

Figure 5. Left, female sand fly midgut lysates separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Blots were incubated with biotinylated *Helix pomatia* agglutinin (HPA) that detects O-glycosylated proteins. Lane 1, molecular mass markers; lanes 2 and 3, *Phlebotomus sergenti*; lanes 4 and 5, *P. arabicus*; +, preincubation of lectin with 250 mmol/L N-acetyl-D-galactosamine; -, no preincubation. Right, reactions of fluorescein-conjugated HPA with *P. arabicus* and *P. sergenti* midgut cells.

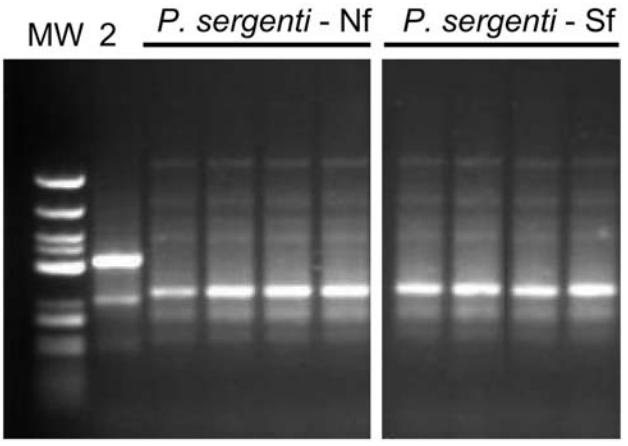

Figure 6. Random amplified polymorphic DNA PCR banding patterns of *Phlebotomus sergenti* from 2 foci in Galilee, Israel. The PCR was performed with primer OPI 1. Lane MW, molecular mass marker; lane 2, *P. sergenti* from Turkey. Shown are 4 flies from the northern focus (Nf) and 4 flies from the southern focus (Sf).

widely distributed in Africa and the Arabian peninsula (*17*), and the Galilee focus forms the northern limit of its distribution. Since *P. arabicus* originates in warmer regions, finding it in warmer, drier, south-facing slopes and not in cooler, shadier north-facing slopes of the hills in Galilee is not surprising (Table 1).

Rock hyraxes in both foci were found infected with *L. tropica*, and 1 isolate was obtained from an adult male in the northern focus. Although rock hyraxes were suspected reservoir hosts of *L. tropica* in Africa (*35*,*36*) and have been previously implicated in the northern focus (*4*), this is the first report of a rock hyrax isolate that was identified as *L. tropica* and shown to be identical to those obtained from humans and sand flies in the same focus (Table 3).

Rock hyrax populations in many parts of Israel are expanding rapidly and encroaching upon human habitation. They were extremely common in both foci studied, as well as in other *L. tropica* foci in the region (D. Meir and A. Warburg, unpub. data; [*4*,*5*]). In the Galilee foci, rock hyraxes inhabit crevices within boulder mounds that were created when land was cleared for the construction of houses. These artificial caves also afford suitable breeding sites for sand flies. Rock hyraxes are susceptible to *L. tropica*, and infected rock hyraxes are infective to feeding *P. arabicus* and *P. sergenti*. Sand flies are attracted to rock hyraxes and prefer feeding on their snouts (Figure 7) (*37*). This behavior makes them suitable as vectors because *L. tropica* is usually found in the skin above the nose (R.W. Ashford, unpub. data). Furthermore, as gregarious diurnal mammals, sleeping rock hyraxes are a readily available blood source for night-questing phlebotomine females. Lastly, rock hyraxes live for 9–10 years in the wild (*38*) and constitute an efficient parasite reservoir for infecting sand flies that emerge in the spring after their winter diapause (*39*). These facts indicate that that burgeoning, peridomestic rock hyrax populations are the primary cause of the emergence of CL caused by *L. tropica* in the region studied (*39*,*40*).

Figure 7. Rock hyrax (*Procavia capensis*). Sand flies are attracted to these animals and prefer feeding on their snouts.

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References

1. Desjeux P. The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg. 2001;95:239–43.
2. Jaffe CL, Baneth G, Abdeen ZA, Schlein Y, Warburg A. Leishmaniasis in Israel and the palestinian authority. Trends Parasitol. 2004;20:328–32.
3. Anis E, Leventhal A, Elkana Y, Wilamowski A, Pener H. Cutaneous leishmaniasis in Israel in the era of changing environment. Public Health Rev. 2001;29:37–47.
4. Jacobson RL, Eisenberger CL, Svobodova M, Baneth G, Sztern J, Carvalho J, et al. Outbreak of cutaneous leishmaniasis in northern Israel. J Infect Dis. 2003;188:1065–73.
5. Schnur LF, Nasereddin A, Eisenberger CL, Jaffe CL, El Fari M, Azmi K, et al. Multifarious characterization of *Leishmania tropica* from a Judean desert focus, exposing intraspecific diversity and incriminating *Phlebotomus sergenti* as its vector. Am J Trop Med Hyg. 2004;70:364–72.
6. Klaus S, Frankenburg S. Cutaneous leishmaniasis in the Middle East. Clin Dermatol. 1999;17:137–41.
7. Kamhawi S, Modi GB, Pimenta PF, Rowton E, Sacks DL. The vectorial competence of *Phlebotomus sergenti* is specific for *Leishmania tropica* and is controlled by species-specific, lipophosphoglycan-mediated midgut attachment. Parasitology. 2000;121:25–33.
8. Pimenta PF, Saraiva EM, Rowton E, Modi GB, Garraway LA, Beverley SM, et al. Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc Natl Acad Sci U S A. 1994;91:9155–6.
9. Sadlova J, Hajmova M, Volf P. *Phlebotomus (Adlerius) halepensis* vector competence for *Leishmania major* and *Le. tropica.* Med Vet Entomol. 2003;17:244–50.
10. Kamhawi S, Ramalho-Ortigao M, Pham VM, Kumar S, Lawyer PG, Turco SJ, et al. A role for insect galectins in parasite survival. Cell. 2004;119:329–41.
11. Warburg A, Tesh RB, McMahon-Pratt D. Studies on the attachment of *Leishmania* flagella to sand fly midgut epithelium. J Protozool. 1989;36:613–7.
12. Hernandez AG, Rodriguez N, Stojanovic D, Candelle D. The localization of a lectin-like component on the *Leishmania* cell surface. Mol Biol Rep. 1986;11:149–53.
13. Svobodova M, Bates PA, Volf P. Detection of lectin activity in *Leishmania* promastigotes and amastigotes. Acta Trop. 1997;68:23–35.
14. Jaffe CL, McMahon-Pratt D. Monoclonal antibodies specific for *Leishmania tropica*. I. Characterization of antigens associated with stage- and species-specific determinants. J Immunol. 1983;131:1987–93.
15. Schonian G, Schnur L, el Fari M, Oskam L, Kolesnikov AA, Sokolowska-Kohler W, et al. Genetic heterogeneity in the species *Leishmania tropica* revealed by different PCR-based methods. Trans R Soc Trop Med Hyg. 2001;95:217–24.
16. Schwenkenbecher JM, Wirth T, Schnur LF, Jaffe CL, Schallig H, Al-Jawabreh A, et al. Microsatellite analysis reveals genetic structure of *Leishmania tropica.* Int J Parasitol. 2006;36:237–46.
17. Artemiev M. Revision of the sandflies of the subgenus *Adlerius* (Diptera Phlebotominae, Phlebotomus). Zool Zh. 1980;59:1177–93.
18. Lewis D. A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). Bulletin of the British Museum (Natural History) Entomology. 1982;45:121–209.
19. Lewis D, Buttiker W. Insects of Saudi Arabia: the taxonomy and distribution of Saudi Arabian *Phlebotomus* sandflies (Diptera: Psychodidae). Fauna of Saudi Arabia. 1982;4:353–83.
20. Aransay AM, Scoulica E, Tselentis Y. Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. Appl Environ Microbiol. 2000;66:1933–8.
21. Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, et al. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003;47:349–58.
22. El Tai NO, El Fari M, Mauricio I, Miles MA, Oskam L, El Safi SH, et al. *Leishmania donovani:* intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. Exp Parasitol. 2001;97:35–44.
23. Schnur LF, Zuckerman A. Leishmanial excreted factor (EF) serotypes in Sudan, Kenya and Ethiopia. Ann Trop Med Parasitol. 1977;71:273–94.
24. Modi GB, Tesh RB. A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. J Med Entomol. 1983;20:568–9.
25. Depaquit J, Ferte H, Leger N, Lefranc F, Alves-Pires C, Hanafi H, et al. ITS 2 sequences heterogeneity in *Phlebotomus sergenti* and *Phlebotomus similis* (Diptera, Psychodidae): possible consequences in their ability to transmit *Leishmania tropica.* Int J Parasitol. 2002;32:1123–31.
26. Kravchenko V, Wasserberg G, Warburg A. Bionomics of phlebotomine sandflies in the Galilee focus of cutaneous leishmaniasis in northern Israel. Med Vet Entomol. 2004;18:418–28.
27. Soares RP, Barron T, McCoy-Simandle K, Svobodova M, Warburg A, Turco SJ. *Leishmania tropica:* intraspecific polymorphisms in lipophosphoglycan correlate with transmission by different *Phlebotomus* species. Exp Parasitol. 2004;107:105–14.
28. Martin-Sanchez J, Gramiccia M, Pesson B, Morillas-Marquez F. Genetic polymorphism in sympatric species of the genus *Phlebotomus*, with special reference to *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera, Phlebotomidae). Parasite. 2000;7:247–54.
29. Depaquit J, Ferte H, Leger N, Killick-Kendrick R, Rioux JA, Killick-Kendrick M, et al. Molecular systematics of the phlebotomine sandflies of the subgenus *Paraphlebotomus* (Diptera, Psychodidae, *Phlebotomus*) based on ITS2 rDNA sequences. Hypotheses of dispersion and speciation. Insect Mol Biol. 2000;9:293–300.
30. Gebre-Michael T, Balkew M, Ali A, Ludovisi A, Gramiccia M. The isolation of *Leishmania tropica* and *L. aethiopica* from *Phlebotomus (Paraphlebotomus)* species (Diptera: Psychodidae) in the Awash Valley, northeastern Ethiopia. Trans R Soc Trop Med Hyg. 2004;98:64–70.

31. Lawyer PG, Mebrahtu YB, Ngumbi PM, Mwanyumba P, Mbugua J, Kiilu G, et al. *Phlebotomus guggisbergi* (Diptera: Psychodidae), a vector of *Leishmania tropica* in Kenya. Am J Trop Med Hyg. 1991;44:290–8.
32. Evangelista LG, Leite AC. Histochemical localization of N-acetylgalactosamine in the midgut *Lutzomyia longipalpis* (Diptera: Psychodidae). J Med Entomol. 2002;39:432–9.
33. Walters LL. *Leishmania* differentiation in natural and unnatural sand fly hosts. J Eukaryot Microbiol. 1993;40:196–206.
34. Pavlicek T, Vivanti S, Fishelson F, Nevo E. Biodiversity and microsite divergence of insects at "Evolution Canyon," Nahal Oren, Mt. Carmel, Israel. II. Orthoptera: Acrididae. Journal of the Entomologic Research Society. 2002;4:25–39.
35. Grove SS. Leishmaniasis in South West Africa/Namibia to date. S Afr Med J. 1989;75:290–2.
36. Sang DK, Njeru WK, Ashford RW. A possible animal reservoir for *Leishmania tropica* s.l. in Kenya. Ann Trop Med Parasitol. 1992;86:311–2.
37. Svobodova M, Volf P, Votypka J. Experimental transmission of *Leishmania tropica* to hyraxes (*Procavia capensis*) by the bite of *Phlebotomus arabicus*. Microbes Infect. 2006;8:1691–4.
38. Fourie PB. The life-span of mammals: estimates for the dassie (*Procavia capensis*). J S Afr Vet Assoc. 1978;49:143–5.
39. Ashford RW. Leishmaniasis reservoirs and their significance in control. Clin Dermatol. 1996;14:523–32.
40. Ashford RW. The leishmaniases as emerging and reemerging zoonoses. Int J Parasitol. 2000;30:1269–81.

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Bartonella quintana Endocarditis in Dogs

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We provide the first evidence that *Bartonella quintana* can infect dogs and cause typical signs of endocarditis. Using PCR and sequencing, we identified *B. quintana* in the blood of a dog from the United States with aortic valve endocarditis and probably also in the mitral valve of a dog from New Zealand with endocarditis.

Bacterial endocarditis is an uncommon, often fatal, disease of dogs (*1*). Although a variety of bacteria can be isolated with routine blood cultures, *Bartonella* spp., gram-negative bacteria with fastidious growth requirements, are the most common etiologic agents (*1*). While *B. vinsonii* subsp. *berkhoffii* (*2*), *B. clarridgeiae* (*3*), and *B. washoensis* (*4*) cause endocarditis in dogs, *B. henselae* and *B. quintana* are the most common species that cause endocarditis in humans (*5*). We provide the first evidence that *B. quintana* can also infect dogs and cause endocarditis.

Case 1

A 3-year-old castrated mixed breed dog was referred to the Veterinary Teaching Hospital of North Carolina State University on December 21, 1999, with lethargy, anorexia, fever, occasional cough, and lameness in the right rear leg of 8 days' duration. The dog was maintained mostly indoors but lived on a farm and was occasionally observed chasing wild animals or fighting with the other 5 dogs in the household. It also had frequent exposure to 2 pet cats. At the time of the dog's illness, all other pets in the household were considered healthy. Physical examination showed a grade 4/6, to-and-fro murmur and changes in the right rear leg compatible with vascular occlusion secondary to thromboembolism. Laboratory abnormalities included marked neutrophilia (43,000/μL, reference range 3,000–11,000/μL) and mild lymphocytosis, eosinophilia, hypoalbuminemia, and hyperglobulinemia. Thoracic radiographs showed mild left atrial enlargement and mild pulmonary interstitial infiltrates. Results of an electrocardiogram were normal, but echocardiography showed a large vegetative lesion on the aortic valve that caused stenosis and severe insufficiency.

After 1 week's treatment with oral amoxicillin-clavulanate, enrofloxacin, enalapril, atenolol, and subcutaneous heparin sodium, the lameness resolved, and the demeanor was normal. Oral aspirin was substituted for heparin, and medications continued for 6 months, at which time the owner reported that the dog was healthy. On reexamination, the murmur was softer (grade 3/6), but the aortic valve vegetation and insufficiency persisted with progressive left atrial and ventricular enlargement. Subsequently, atrial fibrillation developed, and the dog died from refractory congestive heart failure on September 25, 2002. A necropsy was not performed.

Case 2

In November 2003, a 3-year-old castrated mixed breed dog weighing 48 kg was referred to the Veterinary Teaching Hospital of Massey University, Palmerston North, New Zealand, for evaluation of a heart murmur. The dog lived mainly outdoors and had a 1-week history of depression, fever (40.1°C), and swelling of the left tarsus, which resolved with administration of ampicillin and clavulanate. On examination, the dog was febrile (39.8°C) and had marked dyspnea with mild cyanosis. Crackles were heard on both sides of the chest, and a grade 4/6 pansystolic murmur was loudest over the mitral valve area. The dog had numerous fleas (*Ctenocephalides felis*). Laboratory abnormalities included mild nonregenerative

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anemia, mature neutrophilia (23,000/μL, reference range 3,600–11,500/μL), mild hypoalbuminemia, and mildly elevated urea and creatinine levels. The urine was concentrated (1.033) and contained large numbers of granular casts.

The heart appeared normal in thoracic radiographs, but the pulmonary vasculature was mildly enlarged, and a marked diffuse alveolar pattern occurred throughout the lungs. The heart appeared normal on echocardiography.

Despite symptomatic treatment with fluids, furosemide, and amoxicillin-clavulanate, the dog's condition deteriorated rapidly, and the animal was euthanized at the owner's request. Permission was obtained for postmortem examination.

Materials and Methods

Case 1

Routine blood and urine cultures were performed. Specialized blood cultures for *Bartonella* that used blood agar plates and liquid cell culture medium (*6*) were obtained.

A year after the dog died, frozen (–80°C) stored aliquots of whole blood (200 μL) and the culture-negative liquid cell-culture medium (1 mL) were thawed, and DNA was extracted with the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA). PCR was performed with primers that amplify portions of the α-*Proteobacteria* citrate synthase gene (*gltA*) (5′ CAT GCA GAY CAR GAR CAR AAT GCT TCT AC 3′ and 5′ ATW CCN GAA TAA AAR TCA ACA TTN GGR TAH A 3′) and the phage-associated gene (*pap31*) found in several *Bartonella* spp. (Pap31 1(s): 5′ GAC TTC TGT TAT CGC TTT GAT TT 3′ and Pap31 688 (as): 5′ CAC CAC CAG CAA MAT AAG GCA T 3′), as described previously (*7*). With both primer sets, products were amplified by using DNA from the whole blood and the liquid cell-culture medium. The amplicons were cloned with the pGEM-T Easy Vector System (Promega, Madison, WI, USA) and the sequences determined by Davis Sequencing, Inc. (Davis, CA, USA). Sequences obtained were compared with those in GenBank by using AlignX software (Vector NTI Suite 6.0, InforMax, Inc., Invitrogen Corp., Carlsbad, CA, USA).

Case 2

Abnormal tissues found at postmortem examination were fixed in 10% formalin, embedded in paraffin, and sectioned and routinely stained with hematoxylin and eosin. Immunohistochemical testing was performed with rabbit anti–*B. quintana* antibody (1:1,000) and hematoxylin counterstaining as described previously (*8*).

DNA was extracted from the formalin-fixed mitral valve with the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). PCR was performed with primers for *gltA* and the ITS fragment as described previously (*3*,*4*). Also, a 1-step LightCycler nested PCR was performed as previously described (*9*) with external and internal primers amplifying the *fur* gene (*10*). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced with the dRhodamine Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 DNA Sequencer (Applied Biosystems). Multiple alignments were made with the sequences obtained with the CLUSTAL W software, version 1.81 (*11*).

Results

Case 1

Routine blood and urine cultures and specialized blood cultures for *Bartonella* were negative. Amplicons were obtained with primers for the *gltA* (422 bp; GenBank accession no. DQ383817) and the *pap31* (526 bp; GenBank accession no. DQ383818). These sequences had 99% (*gltA*) and 99.8% (*pap 31*) homology with *B. quintana* Fuller (GenBank accession no. BQCSFULLR) and *B. quintana* strain Toulouse (GenBank accession no. BX897700), respectively.

Case 2

On postmortem examination, severe congestion and edema of the lungs with blood-tinged pleural (250 mL) and pericardial (75 mL) effusion were evident. Although the heart was of normal size and shape, multiple soft, friable, irregular red masses, the largest measuring 10 mm in diameter, were firmly attached to 3 cusps of the mitral valve. The aortic valve was normal. Histologic sections of the mitral valve showed multifocal erosions of the endothelium that contained large masses of fibrin admixed with pockets of degenerate neutrophils. While bacteria were not seen in hematoxylin and eosin or gram-stained sections, Warthin-Starry staining showed multiple clusters of rod-shaped organisms within the masses of fibrin. The organisms were also seen by immunohistochemistry with the genus-reactive polyclonal rabbit anti–*B. quintana* antibody and hematoxylin counterstaining (Figure).

The spleen, pancreas, and left kidney had multiple areas of infarction and hemorrhage with numerous intravascular fibrin thrombi. Warthin-Starry–stained sections showed numerous organisms, similar to those found in the valvular masses, within and surrounding many of the thrombosed blood vessels.

No product was obtained with primers for *gltA* and the ITS fragment. Nested PCR that used the *fur* primers, however, did provide a 202-bp amplicon (GenBank accession no. DQ666269) that had 99% homology with *B. quintana*

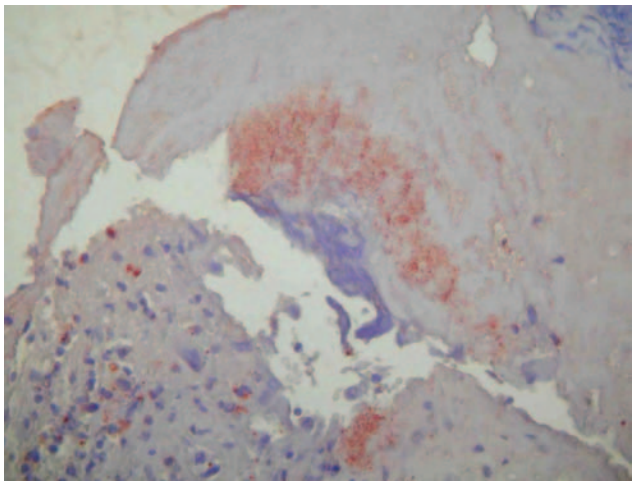

Figure. Immunohistochemical demonstration of bartonellae in the mitral valve with peroxidase-conjugated polyclonal rabbit anti–*Bartonella* sp. antibodies. The organisms stain dark orange against the hematoxylin counterstain; original magnification ×200.

strain Toulouse (GenBank accession no. BX897700) and *B. koehlerae* (GenBank accession no. DQ666271). It had 97% homology with *B. clarridgeiae* strain 94-F40 (GenBank accession no. DQ683729) and lower levels with sequences in GenBank of *B. bacilliformis* (GenBank accession no. AF388198) and other *Bartonella* spp. known to cause endocarditis in dogs and humans: *B. elizabethae* (GenBank accession no. DQ666270), *B. henselae* strain Houston-1 (GenBank accession no. BX897699), and *B. vinsoni* subsp. *berkhoffi* (GenBank accession no. DQ666272).

Discussion

The diagnosis of canine bacterial endocarditis is usually based on appropriate clinical and echocardiographic findings or typical pathologic lesions (*1*). The abnormalities we found were similar to those reported in dogs with bacterial endocarditis and endocarditis due to *Bartonella* spp., namely murmur (89%), fever (72%), leukocytosis (78%), hypoalbuminemia (67%), thrombocytopenia (56%), elevated liver enzymes (56%), lameness (43%), azotemia (33%), respiratory abnormalities (28%), and weakness and collapse (17%) (*1*). One dog had clear echocardiographic evidence of endocarditis; the other had distinct lesions at necropsy not seen with echocardiography.

B. quintana was the most likely cause of endocarditis identified in our dogs. In the first dog, routine blood cultures were negative for other bacteria that cause endocarditis. PCR and sequencing, however, demonstrated DNA of *B. quintana* in the dog's blood at the time endocarditis was diagnosed. Although specialized blood cultures for *Bartonella* spp. were negative, these organisms have fastidious growth requirements, and blood cultures that use solid media have poor diagnostic sensitivity in both humans (*9*) and dogs (*1*).

The most useful techniques for detecting *Bartonella* endocarditis are immunohistochemical analysis of affected valves and PCR (*1*,*5*,*8*). In case 2, the dog had typical histologic lesions of endocarditis that contained large numbers of *Bartonella* organisms, as shown by Warthin-Starry staining and immunohistochemical analysis. When the sequences of the *fur* gene were compared with those of *Bartonella* spp. that are known to cause endocarditis in dogs and humans, the sequencing results showed the *Bartonella* that infected the dog had highest homology (99%) with *B. quintana* and *B. koehlerae*. We did not have control DNA to test for *B. washoensis*, which has been described as an agent of endocarditis in a dog (*4*) and myocarditis in a human (*12*), but we regarded infection with this organism as unlikely because it has only been identified in the United States. Although we know of no specific reports of *B. quintana* in New Zealand, the organism is ubiquitous (*13*) and is the most likely cause of the endocarditis in the dog we studied. We decided the organism was not *B. koehlerae* because it has not been reported in New Zealand or found in recent studies of its natural host (domestic cat) and vector (cat flea) in New Zealand (*14*–*16*). Although the organism causes endocarditis in humans (*17*), it does not appear to be pathogenic in cats, the natural host (*18*).

Our description of *B. quintana* causing disease in the dog is the first report of the organism's pathogenicity in vertebrates other than humans, the natural reservoirs of the organism. Also, our report adds to the growing evidence that *B. quintana* can infect species other than humans. In recent reports, *B. quintana* was identified in a cat euthanized for medical reasons not related to infectious diseases (*19*) and in an apparently healthy captive-bred cynomolgus monkey (*Macaca fascicularis*) (*20*). *B. quintana* was first described as the agent of trench fever in soldiers in World War I. The organism causes a variety of clinical signs, including endocarditis, which is seen most commonly in immunocompetent, homeless men with a history of alcohol abuse (*5*). Although the body louse is the traditional vector of *B. quintana* in humans, this parasite was not a likely source of infection for our dogs since it is host specific, and we found no evidence of infestation. Recent reports of *B. quintana* in persons with no history of body lice have suggested that other vectors may be involved. In France, a high percentages (17%) of *C. felis* contain DNA of *B. quintana*, which suggests that cat fleas might be vectors (*21*). Although the dog from New Zealand had fleas, *B. quintana* has not been identified in *C. felis* in the country (*15*,*16*). Another proposed vector is *Ixodes pacificus* (*22*), but this tick does not occur in North Carolina or New

Zealand. Further, ticks are very rarely found on dogs in New Zealand, where PCR studies on the only prevalent species, *Haemaphysalis longicornis*, have been negative for *Bartonella* spp. (*23*). The source of the *B. quintana* infections in the dogs we describe is unclear.

In summary, our study has shown *B. quintana* can infect dogs and cause endocarditis. Further studies are indicated to investigate the epidemiology of these infections.

Dr Kelly teaches at Ross University Veterinary School in Basseterre, Saint Kitts. His research interest is primarily in vectorborne pathogens.

References

1. MacDonald KA, Chomel BB, Kittleson MD, Kasten RW, Thomas WP, Pesavento P. A prospective study of canine infective endocarditis in northern California (1999–2001): emergence of *Bartonella* as a prevalent etiologic agent. J Vet Intern Med. 2004;18:56–64.
2. Breitschwerdt EB, Atkins CE, Brown TT, Kordick DL, Snyder PS. *Bartonella vinsonii* subsp. *berkhoffii* and related members of the alpha subdivision of the Proteobacteria in dogs with cardiac arrhythmias, endocarditis, or myocarditis. J Clin Microbiol. 1999;37:3618–26.
3. Chomel BB, Mac Donald KA, Kasten RW, Chang CC, Wey AC, Foley JE, et al. Aortic valve endocarditis in a dog due to *Bartonella clarridgeiae*. J Clin Microbiol. 2001;39:3548–54.
4. Chomel BB, Wey AC, Kasten RW. Isolation of *Bartonella washoensis* from a dog with mitral valve endocarditis. J Clin Microbiol. 2003;41:5327–33.
5. Fournier PE, Lelievre H, Eykyn SJ, Mainardi JL, Marrie TJ, Bruneel F, et al. Epidemiologic and clinical characteristics of *Bartonella quintana* and *Bartonella henselae* endocarditis: a study of 48 patients. Medicine. 2001;80:245–51.
6. Maggi RG, Duncan AW, Breitschwerdt EB. A novel chemically modified liquid medium that will support the growth of seven *Bartonella* species. J Clin Microbiol. 2005;43:2651–5.
7. Maggi RG, Chomel B, Hegarty BC, Henn J, Breitschwerdt EB. A *Bartonella vinsonii berkhoffii* typing scheme based upon 16S-23S ITS and Pap31 sequences from dog, coyote, gray fox, and human isolates. Mol Cell Probes. 2006;20:128–34.
8. Lepidi H, Fournier PE, Raoult D. Quantitative analysis of valvular lesions during *Bartonella* endocarditis. Am J Clin Pathol. 2000;114:880–9.
9. Zeaiter Z, Fournier PE, Greub G, Raoult D. Diagnosis of *Bartonella* endocarditis by a real-time nested PCR assay using serum. J Clin Microbiol. 2003;41:919–25.
10. Znazen A, Rolain JM, Hammami N, Kammoun S, Hammami A, Raoult D. High.prevalence of *Bartonella quintana* endocarditis in Sfax, Tunisia. Am J Trop Med Hyg. 2005;72:503–7.
11. Dessen P, Fondrat C, Valencien C, Munier G. BISANCE: a French service for access to biomolecular databases. Comput Appl Biosci. 1990;6:355–6.
12. Kosoy M, Murray M, Gilmore RD Jr, Bai Y, Gage KL. Bartonella strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. J Clin Microbiol. 2003;41:645–50.
13. Maurin M, Raoult D. *Bartonella (Rochalimaea) quintana* infections. Clin Microbiol Rev. 1996;9:273–92.
14. Joseph AK, Wood CW, Robson JM, Paul SL, Morris AJ. *Bartonella henselae* bacteremia in domestic cats from Auckland. N Z Vet J. 1997;45:185–7.
15. Kelly PJ, Meads N, Theobald A, Raoult D. *Rickettsia felis, Bartonella henselae,* and *Bartonella clarridgeiae,* New Zealand. Emerg Infect Dis. 2004;10:967–8.
16. Kelly P, Rolain J-M, Raoult D. Prevalence of human pathogens in cat and dog fleas from New Zealand. N Z Med J. 2005;118:U1754.
17. Avidor B, Graidy M, Efrat G, Leibowitz C, Shapira G, Schattner A, et al. *Bartonella koehlerae,* a new cat-associated agent of culture-negative human endocarditis. J Clin Microbiol. 2004;42:3462–8.
18. Yamamoto K, Chomel BB, Kasten RW, Hew CM, Weber DK, Lee WI, et al. Experimental infection of domestic cats with *Bartonella koehlerae* and comparison of protein and DNA profiles with those of other *Bartonella* species infecting felines. J Clin Microbiol. 2002;40:466–74.
19. La VD, Tran-Hung L, Aboudharam G, Raoult D, Drancourt M. *Bartonella quintana* in domestic cat. Emerg Infect Dis. 2005;11:1287–9.
20. O'Rourke LG, Pitulle C, Hegarty BC, Kraycirik S, Killary KA, Grosenstein P, et al. *Bartonella quintana* in cynomolgus monkey (*Macaca fascicularis*). Emerg Infect Dis. 2005;11:1931–4.
21. Rolain JM, Franc M, Davoust B, Raoult D. Molecular detection of *Bartonella quintana, B. koehlerae, B. henselae, B. clarridgeiae, Rickettsia felis,* and *Wolbachia pipientis* in cat fleas, France. Emerg Infect Dis. 2003;9:338–42.
22. Chang CC, Chomel BB, Kasten RW, Romano V, Tietze N. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. J Clin Microbiol. 2001;39:1221–6.
23. Kelly PJ, Rolain J-M, Midwinter A, Raoult D. Polymerase chain reaction (PCR) survey for rickettsias and bartonellas in ticks from New Zealand. N Z Vet J. 2005;53:468–9.

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Serologic Evidence of Widespread Everglades Virus Activity in Dogs, Florida

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Everglades virus (EVEV), an alphavirus in the Venezuelan equine encephalitis complex, circulates among rodents and vector mosquitoes in Florida and occasionally infects humans. It causes febrile disease, sometimes accompanied by neurologic manifestations. Although previous surveys showed high seroprevalence in humans, EVEV infections may be underdiagnosed because the disease is not severe enough to warrant a clinic visit or the undifferentiated presentations complicate diagnosis. Documented EVEV activity, as recent as 1993, was limited to south Florida. Using dogs as sentinels, a serosurvey was conducted to evaluate whether EVEV circulated recently in Florida and whether EVEV's spatial distribution parallels that of the mosquito vector, *Culex cedecei*. Four percent of dog sera contained neutralizing EVEV antibodies, and many seropositive animals lived farther north than both recorded EVEV activity and the principal vector. These results indicate that EVEV is widespread in Florida and may be an important, unrecognized cause of human illness.

Everglades virus (EVEV), a mosquitoborne Venezuelan equine encephalitis (VEE) complex alphavirus (*Togaviridae*; *Alphavirus*), circulates continuously in enzootic foci in Florida. EVEV infection of humans can result in a nonspecific, flulike, febrile illness that can progress to severe neurologic disease (*1*,*2*). Human EVEV serosurveys in the 1960s and 1970s indicated that people in south Florida were frequently exposed to EVEV. In 1 survey (*3*), >50% of Seminole Indians who resided north of Everglades National Park had antibody to EVEV, and 9% of other groups living in 3 rural communities at the periphery of the park were EVEV seropositive in 1973 (*4*).

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Despite high antibody prevalence, most seropositive persons reported no history of symptoms or signs consistent with VEE-like disease, although exceptions have been noted (*1*,*2*). Among the small number of seropositive persons who experienced illnesses consistent with EVEV infection, the most common signs and symptoms were fever, myalgia, headache, tender lymph nodes, and diarrhea (*4*). Although serologic data indicate that persons are frequently exposed to EVEV, disease is probably most often asymptomatic or not sufficiently severe to require a visit to a physician. In addition, if an EVEV-infected person seeks medical attention, the nonspecific clinical signs and symptoms, similar to those caused by other viral diseases, may not warrant etiologic diagnosis. Persons with undiagnosed diseases of suspected viral etiology are not routinely tested for EVEV by the Florida Department of Health (L.M. Stark, pers. comm.). However, repeated evidence of EVEV antibody in persons living at different locales in south Florida (*3*,*4*) suggests that EVEV may be an unrecognized cause of febrile illness.

All recorded EVEV activity has been limited to south Florida, from Everglades National Park north to Indian River County (*5*) (Figure 1). The last EVEV isolation was reported in 1993 (*6*). As is the case with surveillance for many arboviruses, EVEV activity may be noted only in regions where virologists actively search. No recent serosurveys to detect current EVEV transmission have been performed, and the geographic distribution of EVEV circulation has never been defined by comprehensive surveys. Laboratory susceptibility experiments suggest that *Culex (Melanoconion) cedecei* may be the only important EVEV vector (*7*,*8*). This species has only been reported in 12 southern counties of Florida (*9*), which indicates that if

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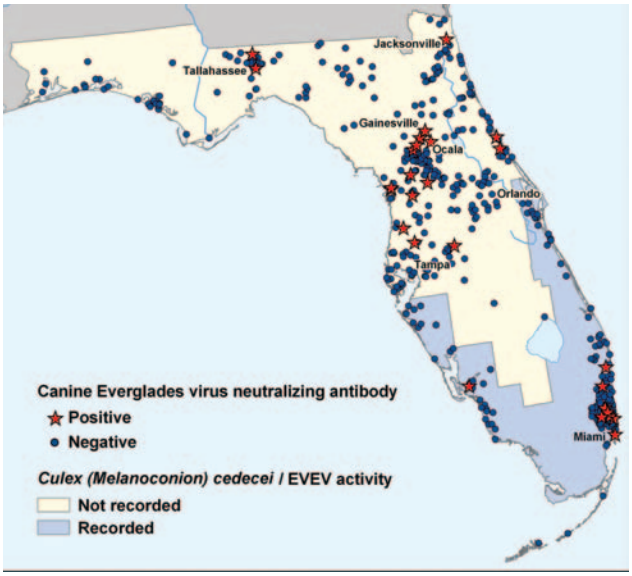

Figure 1. Everglades virus–seropositive and -seronegative dogs in Florida, 2003–2004. A total of 633 samples of dog sera from the Veterinary Medical Center in Gainesville or Hollywood Animal Hospital in Miami were screened. Each blue dot (seronegative) or red star (seropositive) represents a single dog. Most of the seropositive dogs lived in north-central Florida, outside the recorded range of the principal vector *Culex* (*Melanoconion*) *cedecei* or previously recorded Everglades virus activity (purple shading). Owners of dogs living outside the endemic region reported that their animal had not traveled to south Florida.

the mosquito vector regulates EVEV distribution, EVEV activity is probably limited to those areas. Therefore, the goal of this study was to answer 3 questions: 1) Has EVEV recently been circulating in Florida? 2) What is the geographic distribution of EVEV throughout the state? and 3) Does the spatial distribution of EVEV activity, as measured by seroprevalence, parallel the recorded distribution of the principal vector, *Cx. cedecei*?

Testing human sera for EVEV antibody would be an ideal measure of human exposure, but obtaining these samples is difficult from a logistic and regulatory standpoint. Some human pathogen studies have used antibody prevalence of domestic animals to predict human disease risk (*10*–*12*). Canines are effective EVEV sentinels; hemagglutination-inhibiting (HI) and neutralizing antibodies without clinical disease developed in military sentry dogs stationed outdoors in Homestead, Florida (*13*). Dogs experimentally infected with VEEV (strains not reported) survived infection, and minimal HI titers of 320 developed (*14*). Furthermore, beagles exposed to *Aedes triseriatus* mosquitoes infected with the VEEV subtype IAB Trinidad Donkey strain became viremic from days 1 to 5 postinoculation, with virus titers ranging from 1 to 3.8 $\log_{10}$ mouse intraperitoneal median lethal doses per milliliter ($MIPLD_{50}$) (*15*). In a reciprocal study, experimentally infected beagles with viremias of at least 3.7 $\log_{10}$ $MIPLD_{50}$ of VEEV were capable of infecting *Ae. triseriatus* mosquitoes (*16*). Taken together, these results demonstrate that dogs become infected with EVEV or VEEV by artificial and natural inoculation routes; produce viremias of 3–4 days' duration; sustain a nonfatal, febrile infection; and develop detectable antibody.

Field studies in VEEV-enzootic foci outside Florida also indicate that dogs are frequently infected during outbreaks. Domestic dogs tested after epidemics in Colombia (*17*), Venezuela (*18*), and Guatemala (*19*) commonly had neutralizing antibody to VEEV. In these dogs, the average neutralizing antibody titer was lower than titers in experimentally infected canines, possibly reflecting a longer window of time between exposure to VEEV and the time of blood collection, a period during which antibody levels could wane.

Because pet dogs live in close proximity to humans and can serve as effective EVEV sentinels, human exposure to EVEV can be estimated on the basis of dog seroprevalence. Pet dogs are also good sentinels for human arbovirus risk because they more closely approximate the biomass of a human than a hamster or another small mammal, they are restricted to confined geographic zones such as a backyard or neighborhood, and dog owners are knowledgeable about the travel history of their pets. Therefore, we evaluated the distribution of EVEV in Florida by using pet dogs as sentinels of EVEV activity.

Materials and Methods

Serum Collection

Whole blood was collected from pet dogs seen for treatment of various conditions at the University of Florida Veterinary Medical Center in Gainesville, Florida, USA, from July 2003 to January 2004, and at Hollywood Animal Hospital in Hollywood (Miami), Florida, from June to December 2004. Samples from animals living far from Florida, in areas not known to be enzootic for VEEV complex alphaviruses (Galveston, Texas, USA, and Munich, Germany) were kindly provided by resident veterinarians at local clinics and used as negative controls. Dogs from all locations were randomly sampled independent of the reason for the clinic visit. Serum was separated from erythrocytes after low-speed centrifugation. For dogs seen in Gainesville, each pet owner was asked whether the animal had traveled outside of their city of residence, except for the visit to the veterinary clinic.

Antibody Assays

Each serum sample was tested by standard 80% plaque reduction neutralization test (PRNT) (*20*). In brief, neutral-

izing antibody titers were determined by a constant-virus, serum dilution procedure that used Vero (African green monkey kidney) cell monolayers attached to 6- or 12-well plates. Serum samples were heated at 56°C for 30 min for inactivation, and a 1:10 starting serum dilution was serially 2-fold diluted and incubated with an equal volume (250 μL) of ≈800 PFU/mL of EVEV strain FE3–7c for 1 h at 37°C. The virus-serum mixture was incubated onto confluent Vero cell monolayers, overlaid after 1 h with 0.4% agar in Eagle's minimal essential medium and incubated at 37°C for 2 days. The virus was inactivated with 10% formaldehyde, and cell monolayers were stained with 0.05% crystal violet in 30% methanol to visualize plaques. Dilutions of serum that caused a ≥80% reduction in the number of plaques, as compared with negative controls (commercial fetal bovine serum and serum from dogs living outside alphavirus-enzootic areas [Texas and Germany]), were considered positive. Serum from an experimentally infected, EVEV-immune cotton rat was included as a positive control. The reciprocal of the highest dilution of serum (indicated as the final virus-serum dilution) that inhibited at least 80% of plaques was recorded as the antibody titer. To rule out infection with Eastern equine encephalitis virus (EEEV) and Highlands J viruses (HJV), related alphaviruses also enzootic to Florida, all EVEV-seropositive sera were screened by PRNT for antibody to these viruses by using the North American EEEV strain FL-93 (*21*) and HJV strain 86–31227 (*22*).

Location Mapping

To delineate the geographic distribution of antibody to EVEV in canines, the home location of each dog was mapped with ArcGIS for ArcView 9.1 (ESRI, Redlands, CA, USA). Owner street addresses were geocoded (Tele Atlas, Lebanon, NH, USA) and overlaid with land cover and water features (e.g., lakes, ponds, reservoirs, streams, wetlands) from the National Land Cover Dataset (United States Geological Survey, EROS Data Center, Sioux Falls, SD, USA). The proximity of each dog residence relative to water features and forest, agricultural, or suburban or urban land was calculated. The landcover type at the residence location was also determined.

Results

Serology

A total of 633 serum samples from dogs living in Florida were tested for EVEV antibody by PRNT. Of these, 422 were from the Gainesville clinic, and 211 were from the Miami clinic. At least 1 serum sample was obtained from 54 of the 67 counties in Florida, and >20 samples per county were tested from 6 counties, including the greater Miami-Dade area (Broward n = 152, Miami-Dade n = 55, and Palm Beach n = 27) and Ocala region (Marion n = 63). Although the greatest number of samples came from the Miami area, the relative number of dog sera collected per human population by county was highest for Marion County (1 dog per 4,330 people), compared with ratios of 1:45,392 and 1:12,544 in Miami-Dade and Broward Counties, respectively.

Of the 633 sera tested, 26 (4%) contained antibody to EVEV, with 80% PRNT endpoint titers ranging from 20 to 2,560 (Table). None of the EVEV PRNT-positive sera contained detectable neutralizing antibody against EEEV or HJV, which ruled out the possibility of cross-reactions with related, sympatric alphaviruses. None of the dogs from Texas or Germany had detectable antibody to EVEV. The proportion of 80% PRNT-seropositive dogs from Florida (26/633) was significantly greater than that for dogs from Texas and Germany (0/61),which indicated that serosurvey results from Florida dogs were not false positives ($\chi^2 = 2.6$, degrees of freedom [df] = 1, $p<0.05$).

Most of the EVEV-seropositive dogs had PRNT endpoint titers in the low range (20–40). Although the 80% EVEV PRNT titer for 38% of the 26 seropositive dogs was only 20, this value is considered protective for humans

Table. Endpoint plaque reduction neutralization test titers for Everglades virus–seropositive dogs from Florida*†

Approximate age (mo)	80% EVEV PRNT titer	Travel history to south Florida
108	320	No
56	40	No
90	20	No
66	40	No
119	2,560	Resident Miami
67	20	Resident Miami
159	20	Resident Miami
129	160	Resident Miami
9	40	Resident Miami
76	80	Resident Miami
80	20	Resident Miami
130	80	No
121	40	No
78	80	No
80	80	No
140	80	No
62	20	No
132	20	No
86	40	No
58	80	No
62	20	No
101	20	No
114	80	No
95	20	No
114	40	No
18	20	No

*EVEV, Everglades virus; PRNT, plaque reduction neutralization test.
†In addition, an experimentally infected cotton rat had a 5,120 80% EVEV PRNT titer.

vaccinated with the experimental VEEV vaccine and is comparable to levels observed in dogs after outbreaks in Venezuela (*18*). The youngest EVEV antibody–seropositive dog was born in 2003, indicating that EVEV has been circulating recently in the Miami-Dade area.

Geographic EVEV Distribution

A map of the geographic distribution of antibody to EVEV in canines in Florida showed that EVEV-seropositive dogs were spatially dispersed throughout the state (Figure 1). The seropositive dogs resided in major human population centers (Miami, Ocala, and Tallahassee), areas from which more samples were collected because this study focused on domestic canines. Unexpectedly, we observed that EVEV-seropositive dogs lived farther north than the recorded EVEV distribution, which only extends to Indian River County (*9*). Owners of all 16 seropositive dogs from northern and central Florida reported that their pet had not traveled to counties in southern Florida, where EVEV had been previously recorded. The reported *Cx. (Mel.) cedecei* distribution extends only as far north as Brevard County (*9*). The 633 serum samples were grouped on the basis of counties in which EVEV activity or *Cx. cedecei* have been previously recorded (Figure 1). A total of 286 samples were from dogs in counties in which the mosquito vector or EVEV has been recorded ("recorded"), and 347 samples were from dogs in counties where neither the vector nor virus has been recorded ("not recorded"). No significant differences in seroprevalence were found between the recorded group (9/277) and the not-recorded group (18/329) ($\chi^2 = 1.59$, df = 1, $p>0.05$).

Environmental Characteristics of Seropositive-Dog Residences

Figure 2 shows residence locations of EVEV-seropositive dogs overlaid on Florida National Land Cover Data (NLCD) types. Satellite imagery showed that EVEV-seropositive dogs were no more likely to live in rural, forested, or agricultural areas favored by *Cx.* (*Mel.*) *cedecei* than seronegative animals (data not shown). Most (16/26) EVEV-seropositive dogs lived in suburban, urban, or residential environments, a proportion no different from the proportion found for 26 EVEV-seronegative dogs (16/26) chosen by random selection. NLCD data are generally used for broad-scale analysis and do not classify microhabitats. These observations indicate that microhabitats may play a more important role as a predictor of EVEV canine seropositivity than NLCD classifications. In addition to habitat type, no association was found between the location of EVEV-seropositive dogs and average annual precipitation, average annual or minimum temperature, and proximity to bodies of water (data not shown).

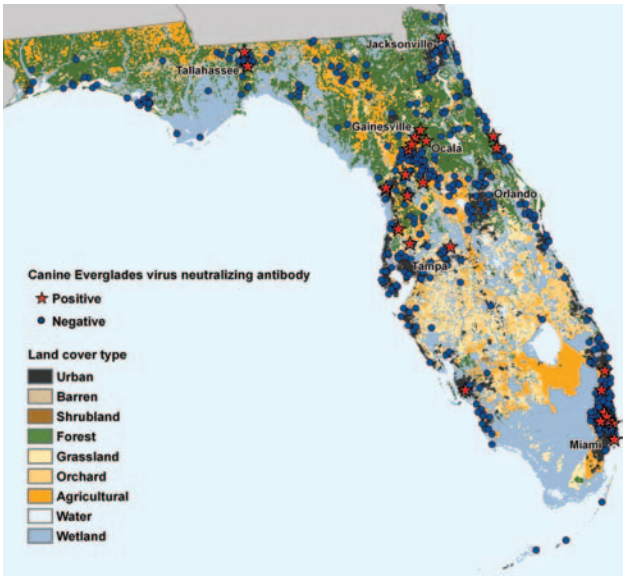

Figure 2. Residence locations of Everglades virus–seropositive and –seronegative dogs in Florida and landcover habitat types obtained from the National Land Cover Database.

Discussion

The objective of this study was to determine the distribution of EVEV activity in Florida on the basis of seroprevalence in pet dogs that live in close proximity to humans. Although virus isolation from mosquitoes or vertebrates would be the most definitive measure of virus activity, attempting to isolate EVEV throughout Florida is logistically challenging and not currently conducted by the state health department. To more precisely determine locations in which canine EVEV infection occurs, seronegative dogs could be housed in specific habitat types in which mosquitoes test positive for EVEV. Other studies that would be useful to corroborate our findings include serosurveys in rodents that serve as EVEV reservoir hosts and that have very limited dispersal ranges. Cattle with known histories have also proven useful as VEEV sentinels (*23*).

EVEV Seropositivity in Northern Florida

Detection of EVEV antibody in dogs living in north and central Florida, without history of travel to south Florida, where EVEV was previously known to be endemic, indicates that EVEV distribution probably extends farther north than previously reported. Possible explanations for this unexpected finding include the following: 1) Dogs residing in the northern parts of Florida became infected during their trip to the veterinary clinic in Gainesville (a clinic visit was common to the history of all of these dogs). We believe that this explanation is highly unlikely because the clinics are indoors and probably are not infested by

many mosquitoes and because even if a dog were bitten by an infected mosquito in or on the way into the clinic, dogs do not seroconvert until several days after infection. 2) Cross-reactive antibodies generated by other alphaviruses endemic to northern Florida were not responsible for EVEV neutralizing antibodies. Overall, 4% of the dog sera we tested contained neutralizing EVEV antibodies, a rate similar to the seroprevalence for the major human pathogenic arboviruses (EVEV, EEEV, Saint Louis encephalitis virus, Western equine encephalitis virus) in Florida detected in >2,500 sera from wildlife in 38 of the state's 67 counties from 1965 to 1974 (*24*). However, alphavirus- neutralizing antibodies generally do not cross-react, and none of the EVEV-seropositive dogs had neutralizing antibodies to EEEV or HJV, which indicates that the dogs sampled were not infected with any known, related, sympatric alphaviruses.

When one considers that pet dogs live close to humans and experience similar mosquito exposure as standard arbovirus sentinels that are housed outdoors continuously in cages (e.g., hamsters, chickens), these results suggest that human infections with EVEV may also occur regularly in many areas of Florida. Therefore, implementing procedures to screen for EVEV in cases of febrile illness or encephalitis might result in recognition of undiagnosed disease.

Our geographic results may have been skewed by the fact that two thirds of the dogs sampled were taken to the referral hospital in Gainesville, potentially oversampling dogs from this region of the state. Despite limitations inherent to using serum samples that were not randomly collected from all areas in Florida, detection of antibody in north and central Florida suggests that the geographic distribution of EVEV is more extensive than previously recorded and extends as far north as Tallahassee. EVEV distribution could also extend outside of Florida, although a serosurvey of raccoons in Georgia did not show EVEV activity that far north (*25*).

Determinants of EVEV Distribution

These results raise a question: What determines the spatial EVEV distribution in Florida? Several hypotheses warrant consideration. First, reservoir host susceptibility and viremia limit the EVEV distribution. The distribution of different cotton rat populations with respect to the known EVEV distribution has not been delineated. However, genetically distinct cotton rats from outside Florida are highly permissive for EVEV viremia (*26*), which diminishes support for this theory. Second, mosquito species other than *Cx*. (*Mel*.) *cedecei* serve as enzootic vectors in northern Florida. The most abundant mammalophilic species in regions of Florida in which EVEV was previously detected, *Aedes taeniorhynchus* and *Cx. nigripalpus*, are not competent EVEV vectors in laboratory experiments (*8*). Although marginally susceptible mosquito species have been implicated as arbovirus vectors during outbreaks when their large population sizes allow for efficient transmission, the species implicated in those settings showed at least some competence in laboratory susceptibility experiments (*27*,*28*). By contrast, *Ae. taeniorhynchus* and *Cx. nigripalpus* were completely refractory to experimental laboratory infection with EVEV (*8*). Also, enzootic viruses in the VEEV complex, including EVEV, are typically highly specific in their use of *Culex. (Melanoconion)* spp. as primary vectors (*29*). Of the 7 vectors of enzootic VEE complex viruses identified to date, all are members of the Spissipes section in the *Culex* (*Melanoconion*) subgenus (*29*). Nevertheless, other species, as well as populations of *Ae. taeniorhynchus* and *Cx. nigripalpus* from northern Florida, should be evaluated in laboratory studies. Field studies should also be conducted since EVEV circulating in northern Florida could be capable of infecting *Ae. taeniorhynchus* or *Cx. nigripalpus*, unlike EVEV isolates and mosquitoes from the Everglades that were used for laboratory vector competence studies. Ticks or other ectoparasites that are widely distributed in Florida could also serve as vectors. Finally, one other possible explanation is that the distribution of EVEV is limited by that of *Cx. cedecei*, but the range of this vector extends beyond that previously recorded or has expanded (or both). Because this species is difficult to identify morphologically, systematic mosquito sampling throughout the state is needed to address this possibility.

Location mapping showed that EVEV-seropositive dogs were not more likely than seronegative dogs to live in environments typically inhabited by *Cx*. *cedecei*. These results should be interpreted with caution because of limitations inherent to using owner reports of pet travel histories. *Cx. cedecei* has been trapped in hardwood hammocks in the Everglades, mangrove swamps, and hardwood forests, but little is known about its proclivity for disturbed or suburban habitats. A closely related VEEV complex virus vector that occurs in Central America, *Cx*. (*Mel.*) *taeniopus* (*30*), is found in high abundance in habitats subject to heavy human disturbance. *Cx. cedecei* might also inhabit suburban or urban areas, but it has not been identified in such environments because of limitations in identification practices.

Clinical Data

In spite of antibody detection in 4% of animals sampled, clinical signs reported for all of the seropositive dogs that were seen at the referral veterinary hospital in Gainesville (detailed data not shown; most animals were diagnosed with tumors or bacterial infections) were inconsistent with EVEV disease in humans (*2*) or laboratory rodents (L.L. Coffey, unpub. data). These observations indicate that EVEV was likely not the underlying cause for

the manifestations seen at these hospitals. Military sentry dogs observed continuously during periods of natural infection, verified by seroconversion, did not show signs of VEE-like illness (*13*).

Despite the low EVEV antibody titers observed in many of the EVEV-seropositive dogs, these results likely represent real infections, especially given that no serum samples from 61 dogs living in areas outside Florida tested positive. Antibody titers in sentry dogs naturally infected with EVEV waned to low levels during a 1- to 2-year period (*13*). The low titers reported here could result from a long interval between infection and blood collection. Alternatively, high antibody titers may never develop in dogs naturally infected with EVEV. For example, 1 young dog born in September 2003 and then sampled <1 year later had a titer of only 40 (Table). The low EVEV antibody titers in naturally infected pet dogs in this study are similar to those in sentry dogs (*13*) but are not as high as titers in dogs experimentally injected with 1,000 suckling mouse $ICLD_{50}$ of EVEV (*31*) or VEE virus (*14*). Higher neutralizing antibody titers might have developed in experimentally infected dogs because the dose of virus administered by intramuscular injection was greater than the amount delivered by a feeding mosquito.

In summary, detection of antibody in dogs throughout Florida suggests that EVEV extends as far north as Tallahassee and has been circulating as recently as 2003. Additional serosurveys involving more dogs, rodents, or both throughout the state, in addition to attempts to isolate EVEV from mosquitoes and vertebrates in regions where seropositive dogs occur, will further define the geographic distribution of EVEV. Enhanced vector surveillance could better define the range of *Cx. cedecei* and may help to explain the unexpected finding of EVEV activity in northern Florida. Screening for EVEV in human patients may also show wide spatial dispersion and a high rate of human infection. EVEV may be a cause of febrile illness or encephalitis in many areas of Florida and should be considered by physicians as a potential cause.

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References

1. Ehrenkranz NJ, Sinclair MC, Buff E, Lyman DO. The natural occurrence of Venezuelan equine encephalitis in the United States. N Engl J Med. 1970;282:298–302.
2. Ehrenkranz NJ, Ventura AK. Venezuelan equine encephalitis virus infection in man. Annu Rev Med. 1974;25:9–14.
3. Work TH. Serological evidence of arbovirus infection in the Seminole Indians of southern Florida. Science. 1964;145:270–2.
4. Ventura AK, Buff EE, Ehrenkranz NJ. Human Venezuelan equine encephalitis virus infection in Florida. Am J Trop Med Hyg. 1974;23:507–12.
5. Day JF, Stark LM, Zhang JT, Ramsey AM, Scott TW. Antibodies to arthropod-borne encephalitis viruses in small mammals from southern Florida. J Wildl Dis. 1996;32:431–6.
6. Mitchell CJ, Morris CD, Smith GC, Karabatsos N, Vanlandingham D, Cody E. Arboviruses associated with mosquitoes from nine Florida counties during 1993. J Am Mosq Control Assoc. 1996;12:255–62.
7. Weaver SC, Scherer WF, Taylor CA, Castello DA, Cupp EW. Laboratory vector competence of *Culex* (*Melanoconion*) *cedecei* for sympatric and allopatric Venezuelan equine encephalomyelitis viruses. Am J Trop Med Hyg. 1986;35:619–23.
8. Coffey LL, Weaver SC. Susceptibility of *Ochlerotatus taeniorhynchus* and *Culex nigripalpus* for Everglades virus. Am J Trop Med Hyg. 2005;73:11–6.
9. Darsie RF, Morris CD. Keys to the adult females and fourth instar larvae of the mosquitoes of Florida (Diptera, Culicidae). Vol. 1. E.O. DeLeon Springs (FL): Painter Printing Company; 2000.
10. Allela L, Bourry O, Pouillot R, Delicat A, Yaba P, Kumulungui B, et al. Ebola virus antibody prevalence in dogs and human risk. Emerg Infect Dis. 2005;11:385–90.
11. Duncan AW, Correa MT, Levine JF, Breitschwerdt EB. The dog as a sentinel for human infection: prevalence of *Borrelia burgdorferi* C6 antibodies in dogs from southeastern and mid-Atlantic states. Vector Borne Zoonotic Dis. 2004;4:221–9.
12. Mannelli A, Mandola M, Pedri P, Tripoli M, Nebbia P. Associations between dogs that were serologically positive for *Rickettsia conorii* relative to the residences of two human cases of Mediterranean spotted fever in Piemonte (Italy). Prev Vet Med. 2003;60:13–26.
13. Nichols JB, Lassing EB, Bigler WJ, Hoff GL. An evaluation of military sentry dogs as a sentinel system to Everglades virus (Venezuelan equine encephalitis FE3–7C strain). Mil Med. 1975;140:710–2.
14. Taber L, Hogge A, McKinney R. Experimental infection of dogs with two strains of Venezuelan equine encephalomyelitis virus. Am J Trop Med Hyg. 1965;14:647–51.
15. Bivin WS, Barry C, Hogge AL, Corristan EC. Mosquito-induced infection with equine encephalomyelitis virus in dogs. Am J Trop Med Hyg. 1967;16:544–7.
16. Davis MH, Hogge AL, Ferrell JF, Corristan EC. Mosquito transmission of Venezuelan equine encephalomyelitis virus from experimentally infected dogs. Am J Trop Med Hyg. 1966;15:227–30.
17. Mackenzie RB. The role of silent vertebrate hosts in epidemics of Venezuelan encephalitis. In: Proceedings of the Workshop-Symposium on Venezuelan Encephalitis Virus. Scientific pub. no. 243, Washington: Regional Office of the World Health Organization; 1972.
18. Dickerman RW, Scherer WF, Navarro E, Ordonez M, Ordonez JV. The involvement of dogs in endemic cycles of Venezuelan encephalitis virus. Am J Epidemiol. 1973;98:311–4.
19. Scherer WF, Ordonez JV, Jahrling PB, Pancake PB, Dickerman RW. Observations of equines, humans and domestic and wild vertebrates during the 1969 equine epizootic and epidemic of Venezuelan encephalitis in Guatemala. Am J Epidemiol. 1972;95:255–66.

20. Beaty BJ, Calisher CH, Shope RE. Arboviruses. In: Emmons RW, editor. Diagnostic procedures for viral, rickettsial and chlamydial infections. 6th edition, Washington: American Public Health Association; 1989. p. 797–855.
21. Aguilar PV, Paessler S, Carrara AS, Baron S, Poast J, Wang E, et al. Variation in interferon sensitivity and induction among strains of eastern equine encephalitis virus. J Virol. 2005;79:11300–10.
22. Cilnis MJ, Kang W, Weaver SC. Genetic conservation of Highlands J viruses. Virology. 1996;218:343–51.
23. Estrada-Franco JG, Navarro-Lopez R, Freier JE, Cordova D, Clements T, Moncayo A, et al. Venezuelan equine encephalitis virus, southern Mexico. Emerg Infect Dis. 2004;10:2113–21.
24. Bigler WJ, Lassing E, Buff E, Lewis AL, Hoff GL. Arbovirus surveillance in Florida: wild vertebrate studies 1965–1974. J Wildl Dis. 1975;11:348–56.
25. Bigler WJ. Serologic evidence of Venezuelan equine encephalitis virus infections in raccoons of south central Florida. J Wildl Dis. 1971;7:166–70.
26. Coffey LL, Carrara A, Paessler S, Haynie M, Bradley R, Tesh R, et al. Experimental Everglades virus infection of the cotton rat (*Sigmodon hispidus*). Emerg Infect Dis. 2004;10:2182–8.
27. Sudia WD, Lord RD, Newhouse VF, Miller DL, Kissling RE. Vector-host studies of an epizootic of Venezuelan equine encephalomyelitis in Guatemala, 1969. Am J Epidemiol. 1971;93:137–43.
28. Miller BR, Monath TP, Tabachnick WJ, Ezike VI. 1989. Epidemic yellow fever caused by an incompetent mosquito vector. Trop Med Parasitol. 1989;40:396–9.
29. Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC. Venezuelan equine encephalitis. Annu Rev Entomol. 2004;49:141–74.
30. Cupp EW, Scherer WF, Lok JB, Brenner RJ, Dziem GM, Ordonez JV. Entomological studies at an enzootic Venezuelan equine encephalitis virus focus in Guatemala, 1977–1980. Am J Trop Med Hyg. 1986;35:851–9.
31. Calisher CH. Silent hosts of Venezuelan equine encephalitis (VEE) virus in endemic situations: mammals. In: Proceedings of the Workshop-Symposium on Venezuelan Encephalitis Virus. Scientific pub. no. 243, Washington: Regional Office of the World Health Organization; 1972.

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Influenza, *Campylobacter* and *Mycoplasma* Infections, and Hospital Admissions for Guillain-Barré Syndrome, England

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Guillain-Barré syndrome (GBS) is the most common cause of acute flaccid paralysis in polio-free regions. Considerable evidence links *Campylobacter* infection with GBS, but evidence that implicates other pathogens as triggers remains scarce. We conducted a time-series analysis to investigate short-term correlations between weekly laboratory-confirmed reports of putative triggering pathogens and weekly hospitalizations for GBS in England from 1993 through 2002. We found a positive association between the numbers of reports of laboratory-confirmed influenza A in any given week and GBS hospitalizations in the same week. Different pathogens may trigger GBS in persons of different ages; among those <35 years, numbers of weekly GBS hospitalizations were associated with weekly *Campylobacter* and *Mycoplasma pneumoniae* reports, whereas among those >35 years, positive associations were with influenza. Further studies should estimate the relative contribution of different pathogens to GBS incidence, overall and by age group, and determine whether influenza is a real trigger for GBS or a marker for influenza vaccination.

Guillain-Barré syndrome (GBS) is the most common cause of acute flaccid paralysis in polio-free regions. Estimated incidence in high-income countries is 0.4–4.0 cases per 100,000 population (*1*). *Campylobacter jejuni* is the most commonly identified infectious trigger for GBS. Several studies have demonstrated evidence of recent *C. jejuni* infection in a higher proportion of GBS case-patients than in controls (*2–10*). Other pathogens, including cytomegalovirus (*7*), Epstein-Barr virus (*7*), *Haemophilus influenzae* (*11–14*), and *Mycoplasma pneumoniae* (*7,15,16*), have been suggested as possible GBS triggers, as was influenza vaccination in the United States during 1976–1977 (*17*). However, epidemiologic evidence that implicates these latter agents remains scarce. We conducted a time-series analysis to investigate temporal associations between weekly variations in reports of microbiologically confirmed infections and hospital admissions for GBS.

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Methods

Reports of Microbiologically Confirmed Infections

Positive microbiologic diagnoses ascertained through voluntary laboratory reporting in England and Wales are recorded in the national infections database (LabBase2) (*18*). We obtained weekly reports of infections suspected of causing GBS, namely, *Campylobacter* spp., cytomegalovirus, Epstein-Barr virus, *Haemophilus influenzae* (B and non-B), *Mycoplasma pneumoniae*, and influenza (A, B, and all influenza) from 1993 through 2002. Influenza vaccination figures are available only quarterly and do not provide sufficient temporal resolution for this analysis.

We used the specimen date for all analyses because onset dates were rarely available. For *Campylobacter*, the median delay between patients' onset date and the specimen date was 4 days (interquartile range 3–7 days); for 90% of cases, the delay was <14 days (*19*). Similar data were unavailable for other pathogens.

GBS Hospitalizations

Nonidentifiable GBS hospitalization data were provided by Hospital Episodes Statistics (HES) (*20*), which

records all in-patient care episodes in English National Health Service hospitals. An episode is a continuous period of treatment under 1 consultant. Each episode includes patient's age, sex, admission date, episode duration, episode number, and ≤14 possible International Classification of Diseases (ICD) diagnoses. Because a patient may see several consultants during a hospital stay, several episodes for the same hospitalization event may appear in HES records. Since April 1997 a unique code identifies episodes for the same patient. A series of continuous episodes constitutes a spell of treatment. This method of episode linkage was applied to records from January 1998 onward. Before then, only episodes classified as first episodes were used to avoid including multiple episodes for the same patient spell.

A GBS admission was defined as a spell with GBS-related ICD codes (ICD-9 357.0/ICD-10 G61.0) in any of the first 3 diagnostic codes. From 1998 through 2002, 3,477 repeat spells were excluded; these are unlikely to represent independent events, e.g., the likelihood of recurrent GBS may depend on host genetic factors.

The 2 series of hospitalizations (1993–1997 and 1998–2002) were collapsed into weekly counts of GBS admissions. For 1993, data were only available from April 1 on. We compared the 2 periods to investigate whether inability to exclude repeat spells from 1993 through 1997 affected the seasonal pattern of GBS admissions. No major differences were seen (Figure 1), and the 2 periods were combined into a weekly time-series of 10 years.

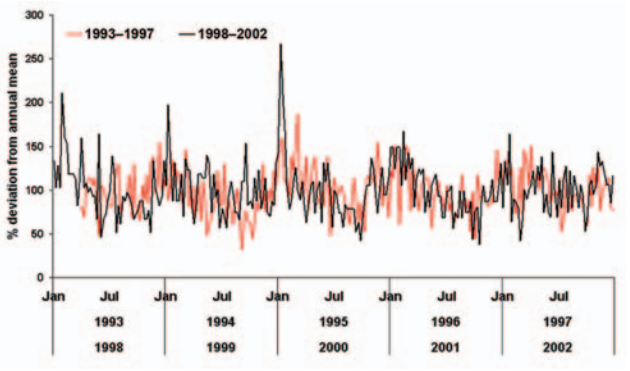

Figure 1. Seasonal distribution of Guillain-Barré syndrome admissions during 1993–1997 and 1998–2002.

Statistical Analysis

We aimed to answer the following question: Is an increase in the number of laboratory reports in any given week associated with increases in GBS hospitalizations in subsequent weeks? In choosing appropriate statistical methods, special characteristics of time-series data must be considered. Such data exhibit nonrandom patterns over time. These include long-term increasing or decreasing trends (whereby weekly GBS hospitalizations within a year are more closely related than between years) and seasonal patterns (whereby the number of GBS hospitalizations in any given week is similar to that in the same week for other years). In addition, weekly hospitalizations are count data, following a Poisson rather than a normal distribution.

For these reasons, time-series observations cannot be considered to be independent, and statistical techniques commonly used for independent, normally distributed data (such as simple correlation) are inappropriate. Special methods that account for temporal dependence in the data are needed. Specifically, temporal dependence in time-series data can result in confounding due to long-term trends (year-on-year) and seasonal (within-year) patterns. Two variables could apparently be related in time because they have similar seasonal characteristics, not because one causes the other. For example, bottled water consumption increases in summer, when the incidence of salmonellosis is highest. This does not imply that bottled water is a risk factor for *Salmonella* infection; rather, bottled water consumption is influenced by ambient temperature, which itself independently influences *Salmonella* transmission. The exposure-outcome association could also be confounded or obscured by other time-varying factors. For example, if influenza causes GBS, GBS admissions should increase in winter, when influenza incidence is highest. However, *Campylobacter* has the opposite seasonality; if *Campylobacter* is also associated with GBS, high numbers of GBS admissions could still occur when influenza reports are low, because these GBS cases are due to *Campylobacter* (or other pathogens with different seasonality). Time-series methods account for such temporal dependencies in data by adjusting for these long-term trends and seasonal patterns, which enables associations to be investigated over shorter periods, independent of trend and seasonal components.

We used multivariable Poisson regression adapted for time-series data (*21–23*) to investigate the effect of weekly variations in reports of different pathogens on the number of GBS hospitalizations; we adjusted for long-term trends and seasonality. The weekly number of GBS hospitalizations was the outcome, and the weekly number of reports for each pathogen was the exposure. We assumed a log-linear relationship between exposure and outcome, i.e., that an increase in the number of reports of a particular pathogen resulted in a constant increase in the log number of GBS hospitalizations throughout the range of laboratory reports.

We adjusted for long-term (year-on-year) trends by including a variable indicating the year of hospitalization in the regression model; thus, we allowed the mean number of GBS hospitalizations to vary between years. We

controlled for seasonality by using Fourier terms (*21*,*24*). Fourier terms can be used to produce a smooth function of expected values for any set of periodic data (e.g., a seasonal pattern). This is achieved by introducing into the regression model a linear combination of pairs of sine and cosine terms (harmonics) of varying wavelengths. A harmonic is an integer fraction of 1 full wavelength (here, 1 year). The more harmonics used, the better the fit to the hospitalization series (i.e., the greater the level of seasonal adjustment). A seasonal pattern with a single peak and single trough within 1 year could be reproduced with 1 harmonic. In reality, seasonal patterns are more complex, and several harmonic terms are required for adequate seasonal adjustment. Given sufficient seasonal adjustment, all variation in the hospitalization series explained by seasonality is removed; any remaining variation must be due to other factors or random noise. This residual variation, independent of long-term trends and seasonal patterns, and its association with weekly reports of infections was our focus of interest. We used 6 harmonics to adjust for periodic patterns in the data >2 months, assuming that GBS risk is increased for ≤2 months after infection. In addition, we introduced a variable that indicated weeks in which public holidays occurred, to adjust for artifactual variation in laboratory reporting and hospitalizations during these weeks.

Lag Effects

Because of the time lag between infection and GBS, the number of GBS admissions is likely to be associated not with the number of laboratory reports in the same week, but with the number of reports some time before. We thus performed separate regressions with exposure variables lagged by ≤8 weeks. Further, because of delays in seeking healthcare, diagnosing infection, and reporting positive diagnoses to national surveillance, increases in hospitalizations could precede increases in laboratory reports. To account for this possibility, we also performed regressions of GBS admissions against laboratory reports within the subsequent 4 weeks.

The core models thus contained the logged GBS hospitalization series as the dependent variable, indicator variables for year, Fourier terms for season, and an indicator variable for weeks with public holidays. The weekly number of reports of a particular pathogen, either in the same week or lagged by a certain number of weeks, was then introduced as the explanatory variable of interest. The regression equation for the models predicting the expectation of the logarithm of weekly GBS hospitalizations, *Y*, was

$$\log(E(Y)) = \alpha + \beta(X_{t-l}^{p}) + \delta_y(year) + S(t) + \phi(holiday) + AR_1$$

where δ_y represents the coefficients for each year *(y)*, *S(t)* represents a smooth function of season (comprising 6 harmonics), and ϕ *(holiday)* is a term representing weeks with public holidays. The regression coefficient, β, is the effect of the exposure of interest (the weekly number of laboratory reports, *X*). Its exponential, the relative risk (RR), reflects the ratio increase in GBS hospitalizations per unit increase in laboratory reports of pathogen *(p)* at lag *t-l*, where *l* ranges from 8 weeks before to 4 weeks after the GBS hospitalization.

We fit separate models for each pathogen at each lag. We assessed model fit by looking at residual variation. We used the partial autocorrelation function (*25*) to investigate the presence of residual autocorrelation, i.e., whether residual variation in GBS hospitalizations in any given week was correlated with residual variation in other weeks. Some degree of autocorrelation at a lag of 1 week remained after adjustment for yearly and seasonal patterns. We controlled for this by adding to all models a term for the residuals lagged by 1 week (a first-order autoregressive term; AR_1 in the equation). The scale parameter for standard errors was set as the Pearson χ^2 statistic divided by the residual degrees of freedom to allow for possible overdispersion in the data.

Age Group Analysis

Associations between GBS and infection could differ between age groups; an association between a pathogen and GBS might only become apparent in a limited age range. We performed subanalyses to investigate associations in different age groups. Because the age distribution of GBS case-patients is not uniform, we categorized age into 3 broad groups: <35 years, 35–64 years, and ≥65 years, according to the age distribution of GBS patient admissions (Figure 2). Age group–specific models were fit similarly to those for all ages.

All lags at which positive associations were found are presented. However, because of the large number of statistical tests performed (13 lags per pathogen per age group), a positive association at a given lag was considered potentially relevant only if it occurred within a cluster. A cluster

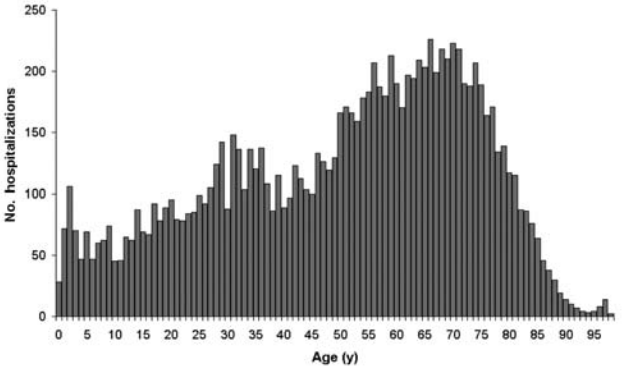

Figure 2. Age distribution of first hospitalization for Guillain-Barré syndrome, England, April 1993–December 2002.

was defined as 2 or more consecutive lags, each associated with the outcome at the 0.05 significance level. This approach reduces the probability of observing chance associations due to multiple testing. Within clusters, lags significant at the 0.01 level were considered important. The adjusted coefficient from these models was used to calculate the expected increase in GBS admissions per 10% increase in the range of laboratory reports at a given lag. All statistical analyses were performed in Stata 8.0 (Stata Corp., College Station, TX, USA).

Results

In the 10-year study period, 11,019 primary admissions for GBS occurred: 2,929 (26.6%) patients were <35 years of age, 4,467 (40.5%) were ages 35–64 years, and 3,623 (32.9%) were ≥65 years. Summary statistics for the weekly number of GBS admissions and laboratory reports for the different pathogens are found in Table 1.

Table 2 gives details of the lags for each pathogen for which significant associations with GBS admissions were found. Only clusters of lags that were significant at the 0.05 significance level are presented. Within clusters, lags that were significant at the 0.01 significance level appear in bold. For example, for influenza reports in all ages, significant associations were found between the number of GBS hospitalizations in any given week and the number of influenza reports in the same week (lag 0) and the previous week (lag 1); the p value for the coefficient at lag 0 was <0.01. Lags that were associated with GBS at the 0.05 significance level but did not occur in clusters are shown in Table 3. Seventeen such lags occurred, consistent with 1 in 20 tests giving a significant result by chance (at $p \leq 0.05$) (given 312 combinations of pathogens, age groups, and lags).

Table 4 presents RRs and 99% confidence intervals (CIs) for the associations shown in Table 2. Only those individual lags from Table 2 that were significant at the 0.01 level of precision are presented. The RRs represent the relative increase in GBS admissions per 10% increase in the range of laboratory reports for a given pathogen at a given lag. For example, for influenza A, the maximum number of laboratory reports in any given week was 398, while the minimum was zero; an increase in influenza A reports of 39.8 (10% of the range) in any given week results, on average, in a 1.03-fold (or 3%) higher incidence of GBS admissions in the same week (RR = 1.032, 99% CI 1.008–1.057). Overall, a positive association was found only with influenza and influenza A at a lag of zero weeks (in the same week as GBS admission).

Different pathogens are associated with GBS admission in different age groups. In those ≤35 years, the number of GBS admissions in a given week was associated with the number of *Campylobacter* spp. reports 5 and 4 weeks earlier and with the number of *M. pneumoniae* reports in the same week and 1 and 3 weeks later.

Among persons ages 35–64 years, a positive association was found between the number of GBS admissions in any given week and the number of all influenza reports 1 and 2 weeks earlier. In those ages ≥65 years, associations were found between the number of GBS admissions and the number of all influenza and influenza A reports in the current week and 1 week before hospitalization.

The results were robust to varying degrees of seasonal adjustment; we repeated the analysis and adjusted for seasonal wavelengths of up to 4 months (3 harmonics) and 1 month (12 harmonics) and used indicator variables for month as well, all with similar results. Table 5 shows those lags that consistently appeared in clusters at all levels of seasonal adjustment. Results for influenza and *M. pneumoniae* were not sensitive to the degree of seasonal adjustment. For *Campylobacter*, clusters of lags were seen with all Fourier models, but not with models that used month indicators.

Table 1. Summary statistics for weekly number of GBS admissions and laboratory reports, England, 1993–2002*

Condition/pathogen	Mean	SD	Median	25th percentile	75th percentile	Minimum	Maximum	10% range
GBS	21.7	6.7	21	17	26	6	51	4.5
Campylobacter spp.	908.2	287.5	875	684	1,104	296	1,737	144.1
Cytomegalovirus	23.5	7.4	22	19	28	6	51	4.5
Epstein-Barr virus	9.9	5.9	10	6	14	0	33	3.3
Haemophilus influenzae non-B	9.2	7.0	7	4	12	0	39	3.9
H. influenzae B	1.0	1.4	0	0	1	0	8	0.8
Mycoplasma pneumoniae	19.6	14.0	15	9	26	1	77	7.6
Influenza	41.4	62.4	13	4	50	0	407	40.7
A	30.9	54.9	9	3	31	0	398	39.8
B	10.4	26.9	2	1	5	0	188	18.8

*GBS, Guillain-Barré syndrome; SD, standard deviation.

Table 2 Poisson regression of Guillain-Barré syndrome (GBS) admissions against infection reports, England, 1993–2002: clusters of lags significant at 0.05 level*†

Pathogen	All ages	<35 y	35–64 y	≥65 y
Campylobacter	–	3,**4**,**5**	–	–
Influenza	**0**,1	–	R1,0,**1**,**2**	R1,**0**,**1**
A	**0**,1	–	–	R1,**0**,**1**
B	–	–	–	–
Mycoplasma pneumoniae	–	R4,**R3**;**R1**,**0**	–	–
Haemophilus influenzae non-B	–	–	–	–
H. influenzae B	–	–	–	–
Cytomegalovirus	–	–	–	–
Epstein-Barr virus	–	–	–	–

*Regression models are adjusted for yearly trend, seasonal pattern (up to 6th harmonic) and public holidays.
†Numbers indicate the lag number in weeks; lag numbers preceded by R represent lags of *n* weeks following the week of admission for GBS. Lags in **boldface** are significant at the 0.01 level of precision.

Discussion

We found associations between the weekly number of laboratory reports of various pathogens and incidence of GBS hospitalizations. Different organisms may be responsible for triggering GBS in different age groups. In particular, *Campylobacter* and *M. pneumoniae* appear to be associated with GBS in those <35 years, while influenza associations were seen in those ≥35 years. Differences in the pathogens responsible for triggering GBS in different age groups have not previously been reported.

No clusters of significant lags were found for cytomegalovirus, Epstein-Barr virus, and *H. influenzae* infections. This could be due to low statistical power (on average, <10 reports per week were made for Epstein-Barr virus and *H. influenzae*), or it could indicate a very small risk or none after these infections.

Our results are subject to several limitations. HES data exclude information from private hospitals. Given the universality of healthcare in England, however, the proportion of GBS cases diagnosed in private hospitals is likely to be small. Although we used only ICD codes specific for GBS, some GBS cases may be classified under nonspecific codes, namely, ICD-9 357.9 (unspecified toxic and inflammatory neuropathy) and ICD-10 G61.9 (inflammatory polyneuropathy unspecified). However, these codes include a large proportion of cases unrelated to GBS; their inclusion in the analysis would dilute any associations with the infections investigated. Misdiagnosis of GBS is also possible; a proportion of GBS diagnoses is likely to represent false positives. We could not validate diagnoses through hospital chart review because identities were hidden in the HES data, and we did not have access to patients' records. However, any misclassification arising from inclusion of false-positive GBS diagnoses will be nondifferential, i.e., the likelihood of misdiagnosis with GBS is unrelated to the likelihood of diagnosis with the pathogens investigated. Inclusion of non-GBS cases could have resulted in effect dilution, but this inclusion would likely not have yielded positive associations when none truly existed.

Laboratory reports for any condition represent only a subset of all symptomatic cases of disease in the community. Our analysis assumes that, for a given condition, the seasonal pattern of laboratory reports accurately reflects the pattern of all community cases. Ascertainment of influenza is likely to be more comprehensive in winter because microbiologic investigation for this pathogen is not routinely conducted outside the influenza season (*26*). This could affect our ability to detect associations in different seasons, but this was not the focus of our study. Our analysis also assumes that the seasonal pattern of laboratory reports is accurately reflected within each age group. This may not be true if, for example, younger persons are less likely to visit the health services (and, thus, be included in

Table 3. Poisson regression of Guillain-Barré syndrome (GBS) admissions against infection reports, England, 1993–2002: unclustered lags*

Pathogen	All ages	<35 y	35–64 y	≥65 y
Campylobacter	–	7	–	–
Influenza	–	–	R3	–
A	–	–	R1,1	–
B	–	–	2,5	–
Myplasma pneumoniae	–	3	–	2
Haemophilus influenzae non-B	8	–	7	–
H. influenzae B	–	5	–	R2
Cytomegalovirus	–	R3,5	–	6
Epstein-Barr virus	–	R1	5	–

*Lags significant at the 0.05 level of precision but not occurring in clusters (regression models are adjusted for yearly trend, seasonal pattern [up to 6th harmonic] and public holidays).

Table 4. Poisson regression of Guillain-Barré syndrome (GBS) admissions against infection reports, England, 1993–2002: regression coefficients*†

Age group/pathogen	Lag no.	10% range in laboratory reports	RR	99% CI	p value
All ages					
Influenza	0	40.7	1.032	1.008–1.057	0.001
Influenza A	0	39.8	1.029	1.006–1.054	0.001
<35 y					
Campylobacter	4	72.0	1.084	1.017–1.156	0.001
	5	72.0	1.074	1.007–1.146	0.004
Mycoplasma pneumoniae	R3	5.6	1.040	1.002–1.079	0.007
	R1	5.6	1.043	1.004–1.083	0.004
	0	5.6	1.041	1.002–1.082	0.006
35–64 y					
Influenza	1	16.9	1.051	1.003–1.102	0.006
	2	16.9	1.047	0.999–1.097	0.011
≥65 y					
Influenza	0	14.8	1.074	1.024–1.126	0.000
	1	14.8	1.051	1.001–1.104	0.008
Influenza A	0	14.3	1.075	1.027–1.126	0.000
	1	14.3	1.052	1.004–1.103	0.005

*Relative risks (RRs) and 99% confidence intervals (CIs) for significant lags by age group and pathogen.
†RRs represent the relative increase in GBS admissions for every 10% increase in the range of laboratory reports for a given pathogen at a given lag.

laboratory reports) for symptoms of influenza during the influenza season. However, laboratory report data for influenza show a distinct and consistent peak during the winter months in all age groups (data not shown).

Among *Campylobacter* spp., only *C. jejuni* is thought to cause GBS. As clinical isolates of *Campylobacter* are not routinely speciated in England and Wales, non-*jejuni* species could not be excluded from the analysis. However, the England and Wales *Campylobacter* Sentinel Surveillance Scheme indicates that 80%–90% of reports of *Campylobacter* infection are due to *C. jejuni* (*19*); inclusion of species not linked to GBS would attenuate rather than inflate any effect on GBS admissions.

The regression coefficients indicate associations between the incidence of various pathogens and GBS admissions, but the coefficients themselves are not directly comparable between pathogens. This is because their magnitude is dependent not only on the true magnitude of the association, but also on the proportion of all cases that is captured by laboratory reports, and this will vary by pathogen (for example, severe conditions are more likely to be reported). Thus, estimates of the relative incidence of GBS due to the different pathogens cannot be obtained from these data. In addition, some evidence exists, particularly for *C. jejuni*, that GBS can develop after subclinical infection. Our analysis did not include asymptomatic infections, so our results apply only to clinical cases of infection. These findings nevertheless raise hypotheses that merit further investigation. For example, several case reports and immunologic analyses have suggested a link between *M. pneumoniae* infection and GBS (*7*,*15*,*16*), but such a link has not been confirmed by robust epidemiologic studies. Our results suggest that studies focusing on younger GBS patients could help clarify any such association.

Whether the associations with influenza are real or whether they reflect seasonal patterns in influenza vaccination is unclear. Influenza vaccination has previously been linked to GBS. During the mass vaccination campaign

Table 5. Poisson regression of Guillain-Barré (GBS) syndrome admissions against infection reports, England, 1993–2002: varying seasonal adjustment*†

Pathogen	All ages	<35 y	35–64 y	≥65 y
Campylobacter	–	–	–	–
Influenza	**0**,1	–	**R1**,0,**1,2**	R1,**0**,1
A	**0**,1	–	–	R1,**0,1**
B	–	–	–	–
Mycoplasma pneumoniae	–	R4,**R3; R1,0**	–	–
Haemophilus. influenzae non-B	–	–	–	–
H. influenzae B	–	–	–	–
Cytomegalovirus	–	–	–	–
Epstein-Barr virus	–	–	–	–

*Significant lags consistently found in clusters with all forms of seasonal adjustment (3, 6, and 12 harmonics and month indicators); regression models are additional adjusted for yearly trend and public holidays.
†Only clusters of lags significant at the 0.05 level of precision are presented. Numbers indicate the lag number in weeks; lag numbers preceded by R represent lags of *n* weeks following the week of admission for GBS. Lags in **boldface** are significant at the 0.01 level of precision.

against swine influenza in the United States during 1976–1977, GBS incidence among vaccinees was 7-fold higher in the 6 weeks after vaccination than in nonvaccinees (*17*,*26*). Similar analyses during subsequent influenza seasons (with no mass vaccination) have found no increased risk (*27*), or a doubling of the risk (*28*), which suggests that differences in antigenic formulation or characteristics of vaccinated populations are influential factors in vaccine-related GBS risk. Here, we found associations with influenza A only; this may reflect the antigenic composition of influenza vaccines, or differential risk resulting from antigenic differences between subtype A and B strains of influenza.

The short lags identified here between increases in influenza reports and subsequent GBS admissions are consistent with a vaccine trigger; the risk period for vaccine-related GBS is believed to be 6 weeks, and increases in vaccination coverage would be expected to precede seasonal rises in influenza. Vaccination could also explain the lack of an association in younger persons, because influenza vaccination is not generally recommended in healthy persons <65 years in the United Kingdom. Influenza vaccine coverage data indicate that for the study period, vaccine uptake was <1% in low-risk groups ages <35 years, <10% among those ages 35–54 years, and 20%–30% among those ages ≥65 years (*29*). For elderly persons at high risk, uptake increased from 40% to 65% from 1993 through 2002 (*30*). These data support the hypothesis that persons in older age groups have a greater vaccine-induced risk of GBS, although a true association with the disease of influenza is still possible. Primary care–based studies investigating the influenza and influenza vaccination status of GBS patients could help resolve this issue.

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References

1. Allos BM. Association between *Campylobacter* infection and Guillain-Barré syndrome. J Infect Dis. 1997;176(Suppl 2):S125–8.
2. Rees JH, Soudain SE, Gregson NA, Hughes RA. *Campylobacter jejuni* infection and Guillain-Barré syndrome. N Engl J Med. 1995;333:1374–9.
3. Enders U, Karch H, Toyka KV, Michels M, Zielasek J, Pette M, et al. The spectrum of immune responses to *Campylobacter jejuni* and glycoconjugates in Guillain-Barré syndrome and in other neuroimmunological disorders. Ann Neurol. 1993;34:136–44.
4. Guarino M, Casmiro M, D'Alessandro R. *Campylobacter jejuni* infection and Guillain-Barré syndrome: a case-control study. Emilia-Romagna Study Group on Clinical and Epidemiological Problems in Neurology. Neuroepidemiology. 1998;17:296–302.
5. Hao Q, Saida T, Kuroki S, Nishimura M, Nukina M, Obayashi H, et al. Antibodies to gangliosides and galactocerebroside in patients with Guillain-Barré syndrome with preceding *Campylobacter jejuni* and other identified infections. J Neuroimmunol. 1998;81:116–26.
6. Ho TW, Mishu B, Li CY, Gao CY, Cornblath DR, Griffin JW, et al. Guillain-Barré syndrome in northern China. Relationship to *Campylobacter jejuni* infection and anti-glycolipid antibodies. Brain. 1995;118:597–605.
7. Jacobs BC, Rothbarth PH, van der Meche FG, Herbrink P, Schmitz PI, de Klerk MA, et al. The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. Neurology. 1998;51:1110–5.
8. Kaldor J, Speed BR. Guillain-Barré syndrome and *Campylobacter jejuni:* a serological study. Br Med J (Clin Res Ed). 1984;288:1867–70.
9. Koga M, Yuki N, Takahashi M, Saito K, Hirata K. Close association of IgA anti-ganglioside antibodies with antecedent *Campylobacter jejuni* infection in Guillain-Barré and Fisher's syndromes. J Neuroimmunol. 1998;81:138–43.
10. Kuroki S, Haruta T, Yoshioka M, Kobayashi Y, Nukina M, Nakanishi H. Guillain-Barré syndrome associated with *Campylobacter* infection. Pediatr Infect Dis J. 1991;10:149–51.
11. Ju YY, Womersley H, Pritchard J, Gray I, Hughes RA, Gregson NA. *Haemophilus influenzae* as a possible cause of Guillain-Barré syndrome. J Neuroimmunol. 2004;149:160–6.
12. Koga M, Gilbert M, Li J, Koike S, Takahashi M, Furukawa K, et al. Antecedent infections in Fisher syndrome: a common pathogenesis of molecular mimicry. Neurology. 2005;64:1605–11.
13. Mori M, Kuwabara S, Miyake M, Dezawa M, Adachi-Usami E, Kuroki H, et al. *Haemophilus influenzae* has a GM1 ganglioside-like structure and elicits Guillain-Barré syndrome. Neurology. 1999;52:1282–4.
14. Mori M, Kuwabara S, Miyake M, Noda M, Kuroki H, Kanno H, et al. *Haemophilus influenzae* infection and Guillain-Barré syndrome. Brain. 2000;123:2171–8.
15. Ang CW, Tio-Gillen AP, Groen J, Herbrink P, Jacobs BC, Van Koningsveld R, et al. Cross-reactive anti-galactocerebroside antibodies and *Mycoplasma pneumoniae* infections in Guillain-Barré syndrome. J Neuroimmunol. 2002;130:179–83.
16. Ginestal RC, Plaza JF, Callejo JM, Rodriguez-Espinosa N, Fernandez-Ruiz LC, Masjuan J. Bilateral optic neuritis and Guillain-Barré syndrome following an acute *Mycoplasma pneumoniae* infection. J Neurol. 2004;251:767–8.
17. Safranek TJ, Lawrence DN, Kurland LT, Culver DH, Wiederholt WC, Hayner NS, et al. Reassessment of the association between Guillain-Barré syndrome and receipt of swine influenza vaccine in 1976–1977: results of a two-state study. Expert Neurology Group. Am J Epidemiol. 1991;133:940–51.

18. Wall PG, de Louvois J, Gilbert RJ, Rowe B. Food poisoning: notifications, laboratory reports, and outbreaks–where do the statistics come from and what do they mean? Commun Dis Rep CDR Rev. 1996;6:R93–100.
19. Gillespie IA, O'Brien SJ, Frost JA, Adak GK, Horby P, Swan AV, et al. A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses. Emerg Infect Dis. 2002;8:937–42.
20. Department of Health (England). Hospital episode statistics [cited 2005 Jan 12]. Available from http://www.hesonline.nhs.uk
21. Schwartz J, Spix C, Touloumi G, Bacharova L, Barumamdzadeh T, le Tertre A, et al. Methodological issues in studies of air pollution and daily counts of deaths or hospital admissions. J Epidemiol Community Health. 1996;50(Suppl 1):S3–11.
22. Schwartz J, Levin R, Goldstein R. Drinking water turbidity and gastrointestinal illness in the elderly of Philadelphia. J Epidemiol Community Health. 2000;54:45–51.
23. Schwartz J, Levin R, Hodge K. Drinking water turbidity and pediatric hospital use for gastrointestinal illness in Philadelphia. Epidemiology. 1997;8:615–20.
24. Vellinga A, Van Loock F. The dioxin crisis as experiment to determine poultry-related campylobacter enteritis. Emerg Infect Dis. 2002;8:19–22.
25. Chatfield C. The analysis of time series: an introduction, 5th ed. London: Chapman and Hall; 1996.
26. Goddard NL, Joseph CA, Zambon M, Nunn M, Fleming D, Watson JM. Influenza surveillance in the United Kingdom: October 2000 to May 2001. [serial online]. Commun Dis Rep CDR. 2001;2005:S1–7.
27. Schonberger LB, Hurwitz ES, Katona P, Holman RC, Bregman DJ. Guillain-Barré syndrome: its epidemiology and associations with influenza vaccination. Ann Neurol. 1981;9(Suppl):31–8.
28. Hurwitz ES, Schonberger LB, Nelson DB, Holman RC. Guillain-Barré syndrome and the 1978–1979 influenza vaccine. N Engl J Med. 1981;304:1557–61.
29. Lasky T, Terracciano GJ, Magder L, Koski CK, Ballesteros M, Nash D, et al. The Guillain-Barré syndrome and the 1992–1993 and 1993–1994 influenza vaccines. N Engl J Med. 1998;339:1797–802.
30. Joseph C, Goddard N, Gelb D. Influenza vaccine uptake and distribution in England and Wales using data from the General Practice Research Database, 1989/90–2003/04. J Public Health (Oxf). 2005;27:371–7.

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Foodborne Transmission of Nipah Virus, Bangladesh

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We investigated an outbreak of encephalitis in Tangail District, Bangladesh. We defined case-patients as persons from the outbreak area in whom fever developed with new onset of seizures or altered mental status from December 15, 2004, through January 31, 2005. Twelve persons met the definition; 11 (92%) died. Serum specimens were available from 3; 2 had immunoglobulin M antibodies against Nipah virus by capture enzyme immunoassay. We enrolled 11 case-patients and 33 neighborhood controls in a case-control study. The only exposure significantly associated with illness was drinking raw date palm sap (64% among case-patients vs. 18% among controls, odds ratio [OR] 7.9, p = 0.01). Fruit bats (*Pteropus giganteus*) are a nuisance to date palm sap collectors because the bats drink from the clay pots used to collect the sap at night. This investigation suggests that Nipah virus was transmitted from *P. giganteus* to persons through drinking fresh date palm sap.

Nipah virus was first recognized in a large human outbreak that affected 283 persons and caused 109 deaths in Malaysia in 1999 (*1*,*2*). The outbreak was preceded by a large Nipah outbreak among pigs (*3*). Contact with sick pigs was the primary risk factor for human Nipah virus infection (*4*). The porcine outbreak, in turn, was thought to be caused by transmission of Nipah virus from fruit bats to pigs. Antibodies against Nipah virus were identified in the 2 native *Pteropus* species (*5*), and the virus was subsequently isolated from pooled urine samples from a *P. hypomelanus* colony on Tioman Island, Malaysia (*6*). The most likely initiating event was that a fruit bat that was shedding Nipah virus in its saliva dropped a piece of partially eaten fruit into a pig sty, and 1 or more of the pigs became infected (*1*,*7*). Genetic characterization of the Nipah virus strains isolated from pigs in the Malaysia outbreak identified 2 strains, 1 of which was identified in humans, and 1 of which may have given rise to the other through genetic drift (*8*). These findings suggest that as few as 1 or 2 instances of spillover of Nipah virus from bats to pigs occurred. No further cases of Nipah virus have been reported in Malaysia since 1999.

Four outbreaks of Nipah virus have been recognized in central and west Bangladesh from 2001 through 2004 (*9*–*11*) (Figure 1). Each outbreak occurred between January and May. Different outbreaks have been associated with different exposures. In the first outbreak in Meherpur in 2001, Nipah case-patients were significantly more likely to have had contact with a sick cow and contact with the secretions of an ill person than were controls (*9*). In Naogaon in 2003, case-patients were more likely than controls to have had contact with a herd of pigs that had passed through the area before the outbreak (*12*). In the 2004 outbreak in Goalando, Nipah case-patients were significantly more likely to have climbed trees and to have had contact with ill persons than were controls (*13*). In the 2004 outbreak in Faridpur, contact with ill persons was the primary risk for human Nipah disease (*10*).

Substantial data implicate flying foxes (*Pteropus* spp.) as the natural reservoir of Nipah virus. Investigations of *Pteropus* spp. in Malaysia, Cambodia, and Thailand have consistently identified antibodies against Nipah virus (*5*, *14*–*16*). It has been isolated from *Pteropus* spp. bats in Malaysia, Cambodia, and Thailand (*6*,*15*,*16*). *P. giganteus* is the only *Pteropus* species present in Bangladesh. In the Naogaon investigation, 2 of 19 *P. giganteus* specimens had

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antibody against Nipah virus. None of 31 other animals from various species had Nipah antibodies (*9*). Strains of Nipah virus isolated from affected persons in Bangladesh have substantial genetic diversity (*17*). The repeated outbreaks in Bangladesh and the genetic diversity of Nipah virus isolated from affected persons in Bangladesh suggest substantial diversity of the virus in the wildlife reservoir and repeated spillover of the virus from its reservoir to the human population.

On January 11, 2005, government health workers in Tangail District reported that 8 previously healthy persons from Basail Upazila (subdistrict) had died within the preceding week from an illness characterized by fever and mental status changes. The Institute for Epidemiology Disease Control and Research (IEDCR) of the government of Bangladesh immediately launched an investigation and 5 days later invited the International Centre for Diarrheal Disease Research, Bangladesh (ICDDRB) to assist. The objectives of the investigation were to determine the cause of the outbreak, identify risk factors for development of illness, and develop strategies for prevention.

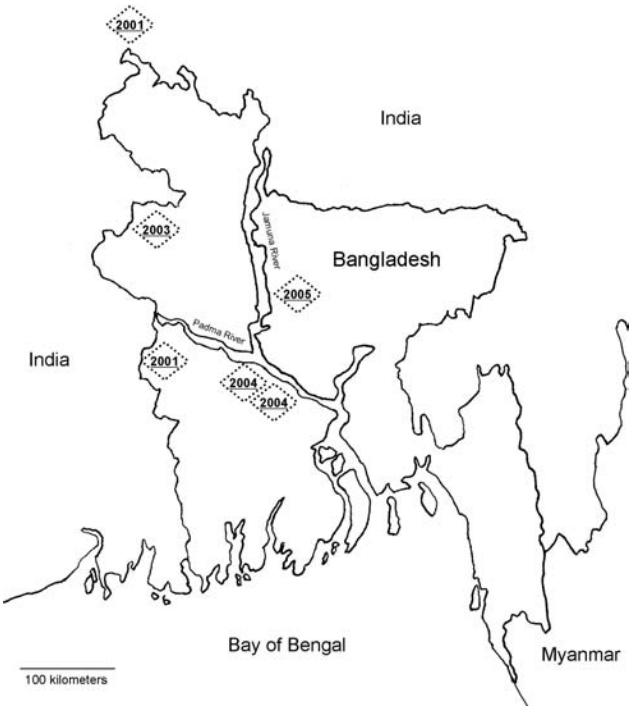

Figure 1. Location and dates of confirmed Nipah virus outbreaks in and near Bangladesh.

Methods

Case Identification

From January 11 onward, government health workers at the Basail Health Center recorded the names and basic clinical information and collected a blood sample from all patients who sought treatment for fever, seizures, or mental status changes. They followed up each ill person at least once per week until he or she recovered. Government health authorities and hospital medical directors in surrounding areas were notified of the outbreak and encouraged to contact the IEDCR if any patients with symptoms of encephalitis from Tangail District sought treatment in a healthcare institution outside of the district. Basail Upazila is composed of 6 unions. Ultimately, the study team defined a case-patient with outbreak-associated encephalitis as a person who lived or traveled in Habla Union, Basail Upazila, Tangail District, Bangladesh, in whom a fever developed and who had new onset of seizures or altered mental status between December 15, 2004, and January 31, 2005.

In-Depth Interviews

An experienced anthropology team conducted in-depth interviews with the families of each case-patient. The objectives were 1) to explore potentially relevant exposures; 2) to assist in framing questions for the case-control questionnaire within the context of the activities, understanding, and language of local residents; and 3) to identify appropriate proxy respondents for each case-patient. Topics covered in the interviews included details on exposure to ill or dead persons; purchase and consumption of date palm sap; contact with animals, especially sick animals; availability and consumption of locally grown fruits, vegetables, and flowering plants; and presence and behavior of fruit bats in the area. The anthropology team interviewed collectors of date palm sap in detail about the context and process of date palm sap collection, preparation of the sap for consumption, and sales and distribution of the sap.

Case-Control Study

On the basis of the findings from the in-depth study, a closed-ended questionnaire was developed and translated into Bengali. Five interviewers with extensive prior experience in administering close-ended questionnaires were trained in a 2-day course on a standardized method to request informed consent and administer the questionnaire. The interviewers then pretested the questionnaire in the presence of supervisors on a sample population from a nearby village that was not included in the study. After final revisions, the interviewers administered the questionnaire to each case-patient or his or her proxy(ies). Controls were identified by visiting the next closest house to the case-patient, confirming that no one in the house met the case definition, identifying the household resident closest in age to the case-patient, and then seeking consent to

administer the questionnaire. Only 1 control was enrolled per household. If the household resident closest in age to the case-patient declined to participate in the study, no other person in the household was sought as a control. This process was repeated at the next closest household until 3 controls were enrolled for each case-patient.

Proxy respondents were identified for each case-patient who had died or was unable to respond. Proxy respondents included spouses, family members, and friends. Multiple proxy respondents were common; for example, a neighborhood playmate could be aware of food exposures that a parent might not be.

Mapping

We measured the location of key features in the outbreak by using global positioning system sensing. We then superimposed these locations on publicly available government maps.

Laboratory Methods

Whole blood specimens were transported on wet ice to the laboratory at ICDDRB, where they were centrifuged; the separated serum was stored at –70°C. Serum samples were shipped on dry ice to the Centers for Disease Control and Prevention (CDC) and tested with an immunoglobulin M (IgM) capture enzyme immunoassay (EIA) that detects Nipah IgM antibodies and an indirect EIA for Nipah IgG antibodies (*18*). Nipah (Malaysia prototype) virus antigen was used in both assays.

Statistics

We used odds ratios to estimate the association of each exposure with disease. To assess whether observations departed from what would be expected by chance, we used the χ^2 test when expected cell sizes were $\geq$5 and the Fisher exact test when expected cell sizes were <5. We calculated exact mid-p 95% confidence limits around the odds ratio. We used an unmatched analysis because neighbors were chosen as controls to ensure that controls arose from the same population as case-patients and not to control for confounding factors. Indeed, all case-patients and controls lived within the same area. We enrolled persons closest in age, not to control confounding by age but rather to provide simple guidelines to the interviewers that would prevent the common tendency to disproportionately enroll heads of households. Only 1 exposure was significantly associated with illness in the initial analysis. To account for the lack of independence among the exposures of the 3 case-patients that occurred in the same household, we used a generalized estimating equations model with an exchangeable correlation matrix (*19*).

Ethics

The investigation team developed messages based on evidence from prior outbreaks on what steps family members could take to prevent person-to-person transmission of Nipah virus. During the outbreak, government health workers actively disseminated these messages. In addition, at the end of each in-depth interview carried out by the anthropology team, messages on steps to prevent person-to-person transmission of Nipah virus were directly communicated to case-patient families. Informed consent was requested of all study participants or their proxies. Because an emergency outbreak investigation was being conducted, the protocol was not reviewed by a human subjects committee.

Results

Government health workers identified 124 persons within the outbreak area in whom fever developed during January 2005. Of these, 12 persons met the outbreak-associated encephalitis case definition. Among case-patients, the most common accompanying symptom was lack of consciousness (Table 1). The patients' median age was 16 years (range 5–85 years); 7 (58%) were male. Eleven (92%) of the persons who met the case definition died. Death occurred a median of 5 days (range 4–9 days) after the first symptom of illness was reported.

The onset of illness for all of the case-patients occurred within 17 days, and all but the last case occurred after 10 days (Figure 2). All of the case-patients lived within 8 km of each other (Figure 3). Three cases occurred in a single household.

Serum specimens were collected from 3 persons who met the outbreak-associated encephalitis case definition (Table 2). Two case-patients had IgM antibodies against Nipah virus by capture EIA. These 2 case-patients had blood collected 8 and 17 days after illness onset. The patient without detectable IgM antibody had his blood collected 2 days after illness onset. Both patients with IgM antibody also had Nipah IgG antibodies detected. Serum was also collected from 20 residents of the outbreak-affected community who had fever but did not meet the outbreak-associated encephalitis case definition. All 20 of these specimens were negative for Nipah IgM and IgG antibodies.

Table 1. Symptoms and signs of persons with outbreak-associated encephalitis, Habla Union, Bangladesh, January 2005

Symptom and signs	No. (%)
Fever	12 (100)
Death	11 (92)
Lack of consciousness	9 (75)
Headache	5 (42)
Vomiting	5 (42)
Seizures	4 (33)
Difficulty breathing	1 (8)

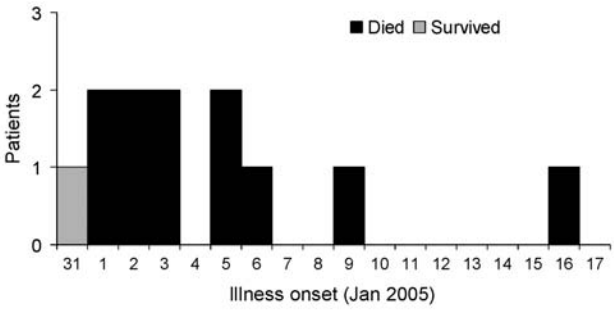

Figure 2. Dates of Illness onset, encephalitis outbreak, Habla Union, Bangladesh.

Case-Control Study

Interviewers enrolled 11 patients who met the outbreak-associated encephalitis case definition and 33 matched controls. One patient was excluded because we could not identify proxy respondents with thorough knowledge of his exposures. Proxy respondents were used for all case-patient interviews and for 6 (17%) control interviews.

The only exposure that was significantly associated with illness was drinking raw date palm sap (64% among cases versus 18% among controls, odds ratio [OR] 7.9, p = 0.01, Table 3). Of the 13 persons who reported consuming date palm sap, 11 knew the location where the sap had been harvested. Ten (91%) reported that the sap was harvested from a single village. None of the study participants were harvesters of date palm sap; none reported drinking the date palm sap directly from the collection container.

A greater proportion of case-patients than controls reported physical contact with sick animals, although this difference may have been due to chance (36% vs. 12%, OR 4.1, p = 0.09). Two case-patients had contact with a sick hen, 1 with a sick cat, and 1 with a sick sheep. None of the 44 case-patients or controls reported physical contact with pigs or fruit bats, and none had eaten bats. Case-patients were no more likely than controls to have climbed trees or to have had contact with ill persons who later died. In the general estimated equation model that adjusted for the 3 cases clustered in the same household, drinking raw date palm sap was significantly associated with illness (adjusted OR 5.6, 95% confidence limits 1.7–7.9, p = 0.03).

Qualitative Findings

Date palm sap collectors explained that harvesting is a seasonal occupation that, in this region, begins in mid-December with the first cold night and continues through mid-February. At the beginning of the season, the bark is shaved off on 1 side of the tree (*Phoenix sylvestris*) near the top in a V shape, and a small hollow bamboo tap is placed at the base of the V. In the late afternoon, date palm sap collectors climb the tree, scrape the area where the bark is denuded so the sap can flow freely, and tie a 2- to 4-L clay pot under the tap. During the night, as the palm sap rises to the top of the tree, some leaks out where the bark is denuded, flows through the tap, and drips into the clay pot. Palm sap collectors climb the trees between 5:00 A.M. and 6:00 A.M. to gather the clay pots.

The date palm sap from the individual clay pots typically contains 1–3 L of sap from a single tree; this sap is poured into a larger metal 22-L aluminum vessel with sap from several trees. Sellers will typically first walk to villages near where they collected the sap and either sell it door to door or from the road. If they still have some

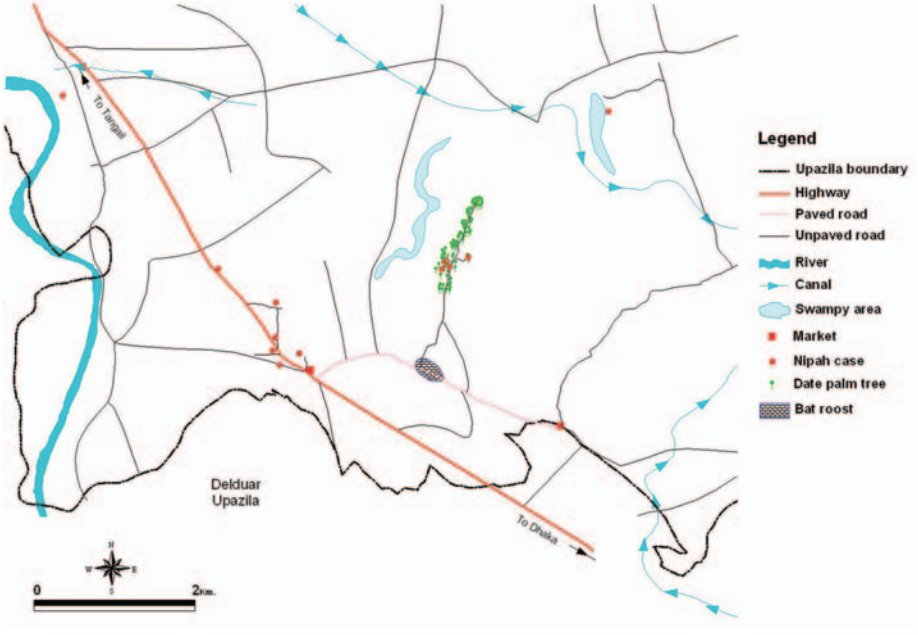

Figure 3. Outbreak area, Habla Union, Basail Upazila.

Table 2. Serum Nipah capture antibody results, encephalitis outbreak, Habla Union, Bangladesh*

Age, y	Illness onset	Date of serum collection	Outcome	Nipah IgM	Nipah IgG
6	Dec 31, 2004	Jan 17, 2005	Survived	+	+
25	Jan 9, 2005	Jan 11, 2005	Died	–	–
12	Jan 16, 2005	Jan 24, 2005	Died	+	+

*Ig, immunoglobulin.

remaining, they will go to the market to sell it. Some buyers cook the sap to make molasses or special deserts; some is consumed fresh. Customers typically bring their own glass or jar, and the date palm sap seller dispenses the sap from the larger container. Fresh sap has to be sold early in the morning; otherwise, the sap will ferment and no longer taste sweet. The market price for fresh date palm sap was 8–10 taka (US $0.14–$0.17) per liter before 10:00 AM and 4–5 taka (US $0.07–$0.09) after 10:00 AM.

Owners of date palm trees reported that they often hear bats at night. Owners viewed the fruit bats as a nuisance because they frequently drink the palm sap directly from the tap or the clay pot. Bat excrement is commonly found on the outside of the clay pot or floating in the sap. Occasionally dead bats are found floating in the pots.

One of the Nipah case-patients who died was the son of a date palm sap collector. The collector harvested sap from his own date palm trees that grew in his household compound, as well from other trees in the area. The collector reported that he had recently heard bats near his trees at night and noted signs of bat excrement in and outside of the clay pot used to collect the sap. His son had been consuming date palm sap on a daily basis since the start of the season. Several days before the outbreak, the date palm sap collector sent a gift of fresh palm sap to his relatives living in a nearby homestead. Encephalitis developed in 3 members of the recipient household; 2 died.

Discussion

This outbreak was almost certainly caused by infection with Nipah virus. The tight clustering of cases in time and space suggests a single etiologic agent. The clinical signs and symptoms of fever, central nervous system involvement, and rapid progression to death are consistent with other Nipah outbreaks in Bangladesh. The outbreak occurred in the same region and during the same time of year as the 4 prior confirmed Nipah outbreaks in Bangladesh. Finally, 2 of 3 persons who met the outbreak-associated encephalitis case definition and were able to provide a serum specimen had IgM antibodies against Nipah virus. The single outbreak-associated specimen that was IgM negative was drawn from a patient on day 2 after symptom onset, so IgM antibodies may not have been present in sufficient quantity to be detected (*20*).

Date palm sap was the likely vehicle of transmission for most of the Nipah virus infections in this outbreak. Drinking fresh, raw date palm sap was the sole exposure significantly associated with illness. Moreover, date palm sap is a biologically plausible vehicle. Although fruit bats uncommonly shed Nipah virus (*5*,*21*), when infected they can shed virus in both saliva and urine (*5*,*15*,*16*). Nipah virus can survive for days in fruit juice or flying-fox urine (*22*). Since date palm sap is sold and consumed within a few hours of collection, consumers could ingest infectious virus.

This outbreak provides further evidence that Nipah virus infection in humans in Bangladesh is a seasonal disease that results from interaction between *P. giganteus* fruit bats and humans. This is the fifth Nipah outbreak in 5 years that has been identified in the same region (Figure 1). A Nipah outbreak also was confirmed in Siliguri, India, 15 km north of the border with Bangladesh in January 2001 (*23*). Each of these outbreaks occurred between January and May. *P. giganteus* is widely distributed throughout Bangladesh (*24*). The reason the outbreaks are occurring in this region at this time of year may be related to a seasonal increase in Nipah virus shedding among *P. giganteus* or to *P. giganteus*' attraction to particular natural or agricultural foods that are seasonally available in this region and bring the bats into proximity with humans.

An important limitation of this investigation is that only 3 serum samples were available from case-patients. Thus, some persons included in the outbreak may not have had Nipah virus infection. Misclassifying non–case-patients as case-patients would bias the odds ratio toward null.

A second limitation is that proxy interviews were required to obtain exposure information for all case-patient interviews. In addition, the same population was used for in-depth interviews and for follow-up quantitative questionnaires. Thus, information bias is possible. However, proxy respondents were independently identified by the qualitative research team to ensure that only persons who directly observed the case-patients' exposures were included. The standardized questionnaire used for the case-control study was pretested. The interviewers were unaware of the study hypothesis, and respondents were encouraged to answer to the best of their knowledge. Discrepancies were found between the in-depth interviews and the quantitative studies. Three proxy respondents who unambiguously reported consuming date palm sap during the initial in-depth qualitative interview reported no consumption during the follow-up close-ended interview. These 3 persons were relatives of a date palm sap collector. Their answers to the case-control questionnaire were used in the analysis.

Table 3. Bivariate analysis of risk factors for encephalitis Habla Union, Tangail District, Bangladesh, 2005

Risk factor	Case-patients with this risk factor, n = 11; no. (%)	Controls with this risk factor, n = 33; no. (%)	Odds ratio	95% Confidence limits*	p value†
Male sex	6 (55)	16 (49)	1.3	0.31– 5.4	0.73
Climbed trees	3 (27)	11(33)	0.8	0.14–3.4	1.0
Physical contact with living animal					
Pig	0	0	Undefined	1.0	
Fruit bat	0	0	Undefined	1.0	
Cow	5 (46)	21 (64)	0.48	0.11–2.0	0.31
Goat	2 (18)	6 (18)	1.00	0.12–5.8	1.0
Sheep	0	2 (6)	0	0, 11	1.0
Chicken	5 (46)	9 (27)	2.2	0.50–9.4	0.29
Duck	3 (27)	7 (21)	1.4	0.24–6.7	0.69
Cat	1 (9)	10 (30)	0.23	0.01–1.7	0.24
Physical contact with any sick animal	4 (36)	4 (12)	4.1	0.7–22	0.09
Physical contact with sick chicken	2 (18)	2 (6)	3.4	0.3–36	0.26
Killed a sick animal	0	1 (3)	0	0–57	1.0
Ate an animal that had been sick at the time it was killed	0	1 (3)	0	0–57	1.0
Drank raw date palm sap	7 (64)	6 (18)	7.9	1.6–38	0.01
Ate					
Banana	3 (27)	11 (33)	0.75	0.14–3.4	1.00
Papaya	1 (9)	7 (21)	0.37	0.01–2.9	0.66
Starfruit	2 (18)	8 (24)	0.7	0.09–3.8	1.0
Guava	5 (46)	14 (42)	1.1	0.27–4.6	1.0
Tamarind	1 (9)	3 (9)	1.0	0.03–11	1.0
Buroy	2 (18)	6 (18)	1.0	0.12–5.8	1.00
Traveled outside subdistrict	4 (36)	10 (30)	1.3	0.28–5.6	0.73
Touched someone with fever and altered mental status who later died	0	7 (21)	0.0	0.0–2.0	0.16
Been in the same room with someone with fever and altered mental status who later died	2 (18)	9 (27)	0.59	0.08–3.2	0.70

*Exact mid-p.
†All p values are 2-tailed. Fisher exact test used when expected cell size <5.

Thus, some study participants probably did realize what some of the investigation team's hypotheses were; however, this factor apparently biased the data against finding an association with date palm sap. Indeed, none of these sources of bias were likely to produce a spurious association between disease and raw date palm sap consumption.

A third limitation is that Nipah virus was not isolated from date palm sap. Indeed, by the time the investigation implicated date palm sap, transmission of Nipah virus was no longer occurring in the area, and we did not collect date palm sap samples. However, the evidence favoring date palm juice as the vehicle for transmission of Nipah virus in this outbreak is stronger than for any alternative hypothesis.

This study highlights the value of a diverse outbreak investigation team. Clinicians identified and cared for ill patients. Experts in qualitative research collaborated with the epidemiology team to understand potential routes of exposure and then used in-depth discussion with affected community residents to identify locally relevant exposures and frame the questions for the quantitative investigation. By conversing with residents of the affected area, qualitative investigators corrected outsiders' misconceptions about local exposures and were also able to quickly develop locally relevant messages to prevent secondary transmission. Laboratory investigators confirmed the cause of the outbreak. A close working relationship between government health workers and researchers permitted shared access to relevant information that provided government authorities with information on how to manage the outbreak and prevent further transmission.

Investigation of different Nipah outbreaks in Bangladesh have identified different routes of transmission including climbing trees, contact with sick persons, and contact with sick animals (*9–13*). This investigation identifies another way that Nipah virus may be transmitted from *P. giganteus* to humans in Bangladesh. Fresh date palm sap is a national delicacy that is enjoyed by millions of Bangladeshis each winter. Apparently, most servings of fresh date palm sap are safe to drink. However, this investigation suggests that, at least occasionally, the sap

contains a sufficient dose of Nipah virus to be fatal to humans. Further research to define how frequently this occurs is important. Persons who want to avoid ingesting Nipah virus from this route, should avoid drinking raw date palm sap. Low-cost interventions to restrict access of fruit bats to date palm taps and pots and that make fresh date palm sap safer should be developed and evaluated. In addition, continued research to better understand Nipah virus transmission between bats and the multiple pathways of human infection are important for developing sound prevention strategies.

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References

1. Chua KB. Nipah virus outbreak in Malaysia. J Clin Virol. 2003;26:265–75.
2. Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A, Lam SK, et al. Nipah virus: a recently emergent deadly paramyxovirus. Science. 2000;288:1432–5.
3. Mohd Nor MN, Gan CH, Ong BL. Nipah virus infection of pigs in peninsular Malaysia. Rev Sci Tech. 2000;19:160–5.
4. Parashar UD, Sunn LM, Ong F, Mounts AW, Arif MT, Ksiazek TG, et al. Case-control study of risk factors for human infection with a new zoonotic paramyxovirus, Nipah virus, during a 1998–1999 outbreak of severe encephalitis in Malaysia. J Infect Dis. 2000;181:1755–9.
5. Yob JM, Field H, Rashdi AM, Morrissy C, van der Heide B, Rota P, et al. Nipah virus infection in bats (order Chiroptera) in peninsular Malaysia. Emerg Infect Dis. 2001; 439–41.
6. Chua KB, Koh CL, Hooi PS, Wee KF, Khong JH, Chua BH, et al. Isolation of Nipah virus from Malaysian Island flying-foxes. Microbes Infect. 2002;4:145–51.
7. Field H, Young P, Yob JM, Mills J, Hall L, Mackenzie J. The natural history of Hendra and Nipah viruses. Microbes Infect. 2001;3: 307–14.
8. AbuBakar S, Chang LY, Ali AR, Sharifah SH, Yusoff K, Zamrod Z. Isolation and molecular identification of Nipah virus from pigs. Emerg Infect Dis. 2004;10:2228–30.
9. Hsu VP, Hossain MH, Parashar UD, Ali MM, Ksiazek TG, Kuzmin I, et al. Nipah virus encephalitis reemergence, Bangladesh. Emerg Infect Dis. 2004;10:2082–7.
10. International Centre for Diarrheal Disease Research, Bangladesh. Nipah encephalitis outbreak over wide area of western Bangladesh, 2004. Health and Science Bulletin. 2004;2:7–11.
11. International Centre for Diarrheal Disease Research, Bangladesh. Person-to-person transmission of Nipah virus during outbreak in Faridpur District, 2004. Health and Science Bulletin. 2004;2:5–9.
12. International Centre for Diarrheal Disease Research, Bangladesh. Outbreaks of encephalitis due to Nipah/Hendra-like viruses. Western Bangladesh. Health and Science Bulletin. 2003;1:1–6.
13. Montgomery J, Hossain MJ, Gurley E, Carroll DS, Croisier A, Bertherat E, et al. Risk factors for Nipah virus infection in Bangladesh: results of a matched case-control study, Emerg Infect Dis. In Press.
14. Daszak P, Plowright R, Epstein JH, Pulliam J, Abdul Rahman S, Field HE, et al. (HERG). The emergence of Nipah and Hendra virus: pathogen dynamics across a wildlife-livestock-human continuum. In: Collinge S, Ray S, editors. Disease ecology: community structure and pathogen dynamics. Oxford (UK): Oxford University Press; 2006, p. 186–201.
15. Reynes JM, Counor D, Ong S, Faure C, Seng V, Molia S, et al. Nipah virus in Lyle's flying foxes, Cambodia. Emerg Infect Dis. 2005;11:1042–7.
16. Wacharapluesadee S, Lumlertdacha B, Boongird K, Wanghongsa S, Chanhome L, Rollin P, et al. Bat Nipah virus, Thailand. Emerg Infect Dis. 2005; 11:1949–51.
17. Harcourt BH, Lowe L, Tamin A, Liu X, Bankamp B, Bowden N, et al. Genetic characterization of Nipah virus, Bangladesh, 2004. Emerg Infect Dis. 2005;11:1594–7.
18. Daniels P, Ksiazek T, Eaton BT. Laboratory diagnosis of Nipah and Hendra virus infections. Microbes Infect. 2001;3:289–95.
19. Hanley JA, Negassa A, Edwardes MD, Forrester JE. Statistical analysis of correlated data using generalized estimating equations: an orientation. Am J Epidemiol. 2003;157:364–75.
20. Ramasundrum V, Tan CT, Chua KB Chong HT, Goh KJ, Chew NK, et al. Kinetics of IgM and IgG seroconversion in Nipah virus infections. Neurol J Southeast Asia. 2000;5:23–8.
21. Chua KB. A novel approach for collecting samples from fruit bats for isolation of infectious agents. Microbes Infect. 2003; 5:487–90.
22. Fogarty R, Halpin K, Mungall BA, Hyatt AD. CSIRO, Geelong, Australia. Effects of environmental conditions on henipavirus survival [abstract 285]. In: Abstracts: International Conference on Emerging Infectious Diseases, 2006 Mar 19–22; Atlanta: Centers for Disease Control and Prevention; 2006.
23. Chadha MS, Comer JA, Lowe L, Rota P, Rollin PE, Bellini WJ, et al. Nipah virus–associated encephalitis outbreak, Siliguri, India. Emerg Infect Dis. 2006;12:235–40.
24. Bates PJJ, Harrison D. Bats of the Indian subcontinent. Sevenoaks (UK): Harrison Zoological Museum; 1997.

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Borrelia lusitaniae and Green Lizards (*Lacerta viridis*), Karst Region, Slovakia

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In Europe, spirochetes within the *Borrelia burgdorferi* sensu lato complex are transmitted by *Ixodes ricinus* ticks. Specific associations are described between reservoir hosts and individual genospecies. We focused on green lizard (*Lacerta viridis*) as a host for ticks and potential host for borreliae. In 2004 and 2005, a total of 146 green lizards infested by ticks were captured, and 469 *I. ricinus* ticks were removed. Borrelial infection was detected in 16.6% of ticks from lizards. Of 102 skin biopsy specimens collected from lizards, 18.6% tested positive. The most frequently detected genospecies was *B. lusitaniae* (77.9%–94.7%). More than 19% of questing *I. ricinus* collected in areas where lizards were sampled tested positive for borreliae. *B. garinii* was the dominant species, and *B. lusitaniae* represented 11.1%. The presence of *B. lusitaniae* in skin biopsy specimens and in ticks that had fed on green lizards implicates this species in the transmission cycle of *B. lusitaniae.*

The causative agents of Lyme borreliosis, spirochetes of the *Borrelia burgdorferi* sensu lato complex, are maintained in natural foci by circulation between the vector ticks in the *Ixodes ricinus* complex and reservoir hosts of various vertebrate taxa. The *B. burgdorferi* s.l. complex encompasses 12 species (*1*–*3*); 4 species have been clearly established as pathogenic to humans: *B. afzelii, B. garinii, B. burgdorferi* s. s., and *B. spielmanii* (*4*–*6*). *B. valaisiana* and *B. lusitaniae,* which were previously considered nonpathogenic, may cause disease as well (*7*,*8*). Different species are associated with distinct ecologic features, levels of pathogenicity, and clinical symptoms in patients.

In Europe, *I. ricinus* ticks infest a wide variety of vertebrate hosts, such as mammals, birds, and lizards. The vertebrate hosts are necessary to maintain the tick population and may also serve as reservoirs for the pathogen. Therefore, the identification of reservoir host species is essential to clarify the transmission patterns of *B. burgdorferi* s.l. in natural foci. The importance of rodents for maintaining *B. afzelii* (*9*), and of birds for *B. garinii* and *B. valaisiana* (*10*), in endemic regions of Slovakia is now indisputable. The National Park Slovak Karst is within the region in which *B. burgdorferi* s.l. in questing ticks and birds has been reported (V. Taragel'ová, unpub. data). In this area, 2 lizard species occur sympatrically, the common wall lizard (*Podarcis muralis*) and the green lizard (*Lacerta viridis*). The green lizard, the dominant species, is frequently infested by immature stages of *I. ricinus* ticks (*11*).

The importance of lizards in the maintenance cycles of *B. burgdorferi* s.l. spirochetes is still controversial. In Italy, *B. lusitaniae* was detected in blood and tissue samples of *P. muralis* (*12*). Furthermore, *Psammodromus algirus,* the most abundant lizard species in North Tunisia, was found to be the primary host for immature stages of *I. ricinus*. Thus, it could play a role in the circulation of borreliae (*13*). *B. burgdorferi* s.s., *B. andersonii,* and *B. bisettii* were detected in the blood of 9 lizard species in the southeastern United States (*14*). On the other hand, several other studies in the United States have shown that the lizards *Sceloporus occidentalis* and *Elgaria multicarinata* are reservoir-incompetent for borreliae because they possess borreliacidal factor in their blood (*15*,*16*). However, 2 lizard species, *Eumeces inexpectatus* and *Anolis carolinensis*, can sustain *B. burgdorferi s. s.* infection (*17*).

In the Slovak Karst (southeastern part of Slovakia), the green lizard is the major host for immature stages of *I. ricinus* ticks (*11*). Therefore, the main aim of this study was to find out whether green lizards can participate in the maintenance cycles of *B. burgdorferi* s.l. in natural foci and

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whether an association with specific borrelial genospecies exists.

Materials and Methods

Study Area

The study was conducted in the National Park Slovak Karst. This area represents a part of the Inner Carpathians in southeastern Slovakia (48°36′ N, 20°52′ E). The climate is warm with low humidity and average temperatures of –4°C in January and 18°C in July. The average rainfall is 700 mm/year.

Tick and Lizard Collection

This survey was conducted in 2004–2005, from May to September, when lizards and ticks are active. Questing *I. ricinus* nymphs and adults were collected by flagging the vegetation in areas where lizards were sampled. Ticks were immediately stored in 70% ethanol.

Green lizards were captured along hiking paths by hand or by noosing, in which a loop made from fishing nylon was attached to the end of a wooden stick and dangled in front of a lizard, which would be captured as it walked through the loop. Animals were characterized by sex and age (adult, subadult, juvenile) and examined for ticks.

Ticks were removed with forceps immediately after capture and stored in 70% ethanol. Biopsy specimens (a 2-cm distal part of the tail and a 1-mm × 1.5-mm piece of skin from collar scales) were taken from each lizard with sterile scissors and put in separate vials with 70% ethanol. Ticks were identified to the species and sex. Only *I. ricinus* ticks were further examined for *B. burgdorferi* sensu lato.

DNA Isolation

Immediately before extraction, ticks and tissues were dried for 30 min to evaporate the ethanol. Each sample was cut with a disposable sterile scalpel. Tissue DNA from lizards' tails and scales was extracted by using DNeasy tissue kit (Qiagen, Hilden, Germany). Extraction steps were conducted according to the manufacturer's protocol. Genomic DNA from ticks was isolated by alkaline hydrolysis (*18*). Incubation time was extended from 5 to 30 min. Isolated DNA was stored at –20°C.

PCR

PCR amplification was performed in a 25-μL reaction mixture from the MasterTaq DNA polymerase kit (Eppendorf AG, Hamburg, Germany) containing 10.4 μL deionized water, 5 μL 5× TaqMaster PCR Enhancer, 2.5 μL 10× Taq buffer (with 15 mmol/L Mg^{2+}), 1.5 μL 25-mmol/L solution of Mg $(OAc)_2$, 0.1 μL Taq DNA polymerase (5 U/μL), 0.5 μL deoxynucleoside triphosphate (dNTP) mix (10 mmol/L) (Fermentas, Vilnius, Lithuania), 1.25 μL of each primer (10 pmol/μL) (Invitrogen, Paisley, Scotland), and 2.5 μL DNA template.

To verify that DNA had been successfully isolated from each tick, primers for the fragment of the tick's mitochondrial cytochrome *b* gene (620 bp) were used (*19*). Negative samples were excluded from the further analysis. Positive samples were examined for the presence of *B. burgdorferi* s. l. by amplifying a portion of the 5S (*rrfA*)-23S (*rrlB*) rDNA intergenic spacer (*20*). PCR products were subjected to electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator.

RFLP Analysis

The positive PCR products of the 5S-23S rDNA intergenic spacer regions were further analyzed by restriction fragment length polymorphism (RFLP). Previously extracted DNA of *B. afzelii, B. garinii, B. valaisiana*, and *B. burgdorferi* s.s. were used as positive controls. For each positive sample, 13 μL amplified DNA was digested at 65°C overnight in a solution containing 5 U of *Tru1*I (300 U/mL) and 1× Buffer R (Fermentas). Electrophoresis was conducted in 16% polyacrylamide gel at 150 V for 3 h. The gels were stained with SYBR gold nucleic acid gel stain (Molecular Probes, Leiden, the Netherlands) for 20 min, and bands were visualized with a UV transilluminator. RFLP profiles that differed from the known profiles of positive controls were further analyzed by sequence analysis.

DNA Sequencing of PCR Products

Sequencing was performed at the Department of Molecular Biology (Faculty of Natural Sciences Commenius University, Bratislava, Slovak Republic). PCR of the 5S-23S rDNA intergenic spacer was conducted according to the protocol described above. For the *fla* gene amplicons, DNA strands were sequenced as described previously (*21*). PCR products were purified by using a QIAquick PCR purification kit (Qiagen). The complementary strands of each sequenced product were manually assembled. Sequences were compared with GenBank entries by Blast N2.2.13 (*22*). Homologous sequences were aligned by using the CLUSTAL W Multiple Sequence Alignment Program (version 1.81) (*23*). Sequence similarity among the sequences were calculated by EMBOSS Align, a pairwise alignment algorithm (http://www.ebi.ac.uk/emboss/align).

The accession numbers of 5S-23S rDNA intergenic spacer sequences obtained in this study are DQ539339 and DQ539340. Accession numbers of flagellin sequences obtained in this study are DQ788618, DQ788619, and DQ788620.

Data Analysis and Statistics

To estimate the probability of a tick's becoming infected after engorging on a green lizard and to measure the degree of infectiousness of infected animals, specific infectivity I_s (*24*) and transmission coeficient $\beta_{H\text{-}T}$ (*9*) were calculated. Individual infectivity (i) is defined as the proportion of larvae derived from an individual lizard that are infected ($i = l_i/l_{h,}$ l_i is the number of larvae that become infected, and l_h is the total number of larvae derived from that host). The specific infectivity (I_s) of a reservoir host species is defined as the sum of individual infectivities and number of individual lizards sampled ($I_s = \Sigma i_S/n_{S,}$ n is the number of individual ticks captured). The host-to-tick transmission coefficient (β_{H-T}) is defined as the portion of the sum of individual infectivities and the number of lizards that infected ≥1 larva ($\beta_{H-T} = \Sigma i_S/n_{iS}$ (n_i is the number of individual hosts that gave rise to at least 1 infected tick). Differences in the prevalence of *B. burgdorferi* s.l. in *I. ricinus* were evaluated statistically with the 2-tailed χ^2 test (degrees of freedom [df] = 1). A value of $p \leq 0.05$ was considered statistically significant.

Results

Lizards and Infestation with Ticks

One hundred forty-six (84 male, 52 female, and 10 subadult) of 165 (89 male, 61 female, and 15 subadult) captured green lizards were infested by ticks during the study period**.** In total, 469 (199 larvae and 270 nymphs) ticks were removed and further identified as *I. ricinus*. Male lizards were infested with 363 ticks (131 larvae and 232 nymphs), which represented 77.4% of all collected ticks. Moreover, 53 tails and 102 skin biopsy specimens were taken from the captured lizards.

B. burgdorferi Prevalence in Ticks Collected from Lizards

DNA isolation was successful in 464 ticks (197 larvae and 267 nymphs), from which the fragment of cytochrome *b* gene was amplified. These ticks were further analyzed for the presence of *B. burgdorferi* s.l. In total, 77 (16.6%) ticks carried borreliae. The infection prevalence between nymphs (15.2%) and larvae (17.6%) did not differ significantly ($p = 0.49669$, df = 1) (Table 1). Twenty-nine percent of tick-infested lizards carried ≥1 infected tick. Infected lizards yielded ≈2 infected larvae per host.

Genotyping with PCR-RFLP identified the following species: *B. lusitaniae, B. afzelii, B. garinii,B. burgdorferi s.s.*, and *B. valaisiana.* Of the *B. burgdorferi*–positive ticks, most (77.9%) were infected with *B. lusitaniae*. The presence of this species was significantly higher than that of other species ($p \leq 0.001$). *B. lusitaniae* was detected in 26 (86.7%) larvae. *B. afzelii, B. garinii,* and *B. burgdorferi* s. s. each were found in 1 larva. Of the 47 *B. burgdorferi*–infected nymphs, 34 (72.3%) were infected with *B. lusitaniae,* 5 (10.6%) with *B. afzelii,* 2 (4.3%) with *B. burgdorferi* s. s., and 1 (2.1%) with *B. garinii*. A mixed infection of *B. lusitaniae* and *B. burgdorferi* sensu stricto was found in 5 (10.6%) nymphs (Table 1). Nymphs and larvae did not differ significantly in the prevalence of *B. lusitaniae*.

Male lizards were parasitized by 61 (79.2%) of 77 infected ticks. Variability of detected genospecies was higher in ticks collected from male than from female lizards. Larvae that fed on female lizards were only infected with *B. lusitaniae*. Out of 7 infected nymphs collected from females, *B. lusitaniae* was present in 5 and *B. afzelii* in 1 tick; *B. lusitaniae* and *B. burgdorferi* s.s. were detected as mixed infection in 1 nymph. The specific infectivity from lizards to larval ticks was highest for *B. lusitaniae*. The specific infectivity of female lizards was slightly higher than that of males (Table 2).

B. burgdorferi Prevalence in Lizards

Isolated genomic DNA from tails and skin biopsy specimens from collar scales was tested for the presence of *B. burgdorferi* sensu lato. None of 53 tested tail samples was positive. Of 102 skin biopsy specimens collected from green lizards, 19 (18.6%) tested positive. Differences in infection prevalence between sexes (18.2% in males vs. 23.7% in females) were not significant. Of 9 skin biopsy specimens from subadult individual lizards, 2 (22.2%) were borrelia positive. The most frequently detected genospecies was *B. lusitaniae* (94.7%), which was present in 18 samples. One lizard was infected with *B. afzelii*.

B. burgdorferi Prevalence in Questing Ticks

Cytochrome *b* was amplified in 325 of 331 (71 female, 73 male, and 187 nymph) questing ticks. Therefore, only these 325 ticks (71 female, 71 male, and 183 nymph) were analyzed further for the presence of *B. burgdorferi* s.l. Sixty-three (19.3%) ticks tested positive. *B. burgdorferi*

Table 1. Variability of *Borrelia burgdorferi* sensu lato in ticks collected on lizards

Stage	No. ticks examined	No positive ticks (%)	No. ticks positive for genospecies (% positive ticks)					
			B. lusitaniae	*B. afzelii*	*B. garinii*	*B. burgdorferi* s.s.	*B. valaisiana*	*B. lusitaniae* + *B. burgdorferi* s.s.
Larvae	197	30 (15.2)	26 (86.7)	1 (3.3)	1 (3.3)	1 (3.3)	1 (3.3)	0
Nymphs	267	47 (17.6)	34 (74.5)	5 (10.6)	1 (2.1)	2 (4.3)	0	5 (10.6)
Total	464	77 (16.6)	60 (77.9)	6 (7.8)	2 (2.6)	1 (1.3)	1 (1.3)	5 (6.5)

Table 2. Specific infectivity (I_s) and host-to-tick transmission coefficient (β_{H-T})

	Males		Females		Total	
Genospecies	I_s	β_{H-T}	I_s	β_{H-T}	I_s	β_{H-T}
Borrelia burgdorferi s.l.	0.0477	0.605	0.0221	0.571	0.0697	0.5753
B. lusitaniae	0.0377	0.518	0.0221	0.571	0.0597	0.5263
B. garinii	0.0012	0.01	–	–	0.0012	0.01
B. afzelii	0.0012	0.01	–	–	0.0012	0.01
B. burgdorferi s.s.	0.0012	0.01	–	–	0.0012	0.01

prevalence in female ticks was the same as in male ticks (22.5%), and it was lower in nymphs (16.9%).

RFLP analysis of the amplified products resulted in 5 distinct profiles. Of the 63 positive ticks, 21 (33.3%) were infected with *B. garinii*, 19 (30.2%) were infected with *B. afzelii*, 8 (12.7%) were infected with *B. burgdorferi* s. s., 7 (11.1%) were infected with *B. lusitaniae*, and 7 (11.1%) were infected with *B. valaisiana*. One nymph was infected simultaneously with *B. garinii* and *B. valaisiana* (Table 3).

Sequence Analysis

Representative samples of RFLP profiles that were different from the known profiles of positive controls were sequenced. The fragment of the 5S-23S rDNA intergenic spacer obtained from the *B. burgdorferi* s.l.–positive nymph (538N) from lizard belonged to *B. lusitaniae*. It was 100% identical to a *Borrelia*-positive skin biopsy specimen (277S) sampled from a lizard. Both obtained sequences were 100% identical with the Turkish *B. lusitaniae* strain Tr213 (AB 091802) and 98.9%, 98.4%, and 94.5% similar to PotiBL37 (AY 463167), PotiB2 (L30131), and PotiB3 (L30132) strains from Portugal, respectively. To better characterize *B. lusitaniae* circulating in ticks and lizards from Slovak Karst, the *fla* gene from a *B. burgdorferi* s. l.–positive questing adult tick and skin biopsy specimen from collar scale was amplified and sequenced. The flagellin sequence of *B. lusitaniae* detected in a skin biopsy specimen (277 S) was 100% identical and 99.6% similar to *B. lusitaniae* detected in questing adult ticks (43 ZLIF, 47 ZMLIM), respectively. Genotypes 277S and 43ZLIF were 100% identical with the Turkish *B. lusitaniae* strain Tr213 (AB091812) as well as with the Polish strain D23–04 (DQ 016623). Genotype 47ZLIM was 99.6%, 99.6%, and 99.4% similar to Tr213, D23–04, and PotiB2 (DQ111036), respectively.

Discussion

The role of lizard species in maintaining *B. burgdorferi* s.l. has not been clearly elucidated yet. In United States, some lizard species have sustained borrelial infection (*14*,*17*); however, other species are incompetent reservoir hosts (*15*,*16*). The reservoir competence of lizards seems to be species specific. Therefore the aim of our study was to establish whether a relationship exists between green lizards, the dominant lizard species in the Slovak Karst, and *B. burgdorferi* s.l., which circulates in this area.

Seventeen percent of ticks that fed on lizards were infected with *B. burgdorferi* s.l. Seventy-eight percent of all infected ticks were infected with *B. lusitaniae*. Moreover, 18.6% of skin biopsy specimens from lizards were positive for *B. burgdorferi* s.l., and almost all (94.7%) were infected with *B. lusitaniae*. Similarly, *B. lusitaniae* have been detected in blood and tissue samples of *Podarcis muralis* in Tuscany in Italy, where borreliae were detected in 2 of 14 tested whole tails from lizards (*12*). At the beginning of our study, we also collected the distal tip of a lizard's tail because this method is minimally invasive and convenient for obtaining a tissue sample. The tissue at the tail, however, is squamous and keratinized, and none of the collected samples was borreliae positive. Therefore, we also obtained skin biopsy specimens from collar scales. These are elongated and extend from the skin on the ventral side, so collecting them is minimally invasive and perhaps more likely to detect infection with *B. burgdorferi* s.l. because most of the immature *I. ricinus* ticks parasitize at the dorsal area (pers. observation). Furthermore, collar scales were chosen to avoid detecting the borreliae that persist in the skin after feeding of the infected ticks, which may enable infection of ticks by "extended co-feeding" (*25*). In this manner, incompetent host species may contribute to the circulation of *B. burgdorferi* s.l. in nature. For example, in England,

Table 3. Variability of *Borrelia burgdorferi* sensu lato in questing ticks

			No. ticks positive for genospecies (% of positive ticks)					
Stage	No. ticks examined	No. positive ticks (%)	*B. afzelii*	*B. garinii*	*B. valaisiana*	*B. burgdorferi* s.s.	*B. lusitaniae*	*B. valaisiana* + *B .garinii*
Nymphs	183	31 (19.6)	13 (41.9)	12 (38.7)	2 (6.4)	3 (9.6)	0 (0)	1 (3.2)
Females	71	16 (22.5)	4 (25)	4 (25)	3 (18.7)	3 (18.7)	2 (12.5)	0 (0)
Males	71	16 (22.5)	2 (12.5)	5 (31.3)	2 (12.5)	2 (12.5)	5 (31.3)	0 (0)
Total	325	63 (19.3)	19 (30.2)	21 (33.3)	7 (11.1)	8 (12.6)	7 (11.1)	1 (1.5)

I. ricinus ticks cofeeding on sheep become infected with *B. burgdorferi*, although sheep themselves are refractory to infection (*26*). In Europe the principal importance of cofeeding to Lyme disease ecology has been suggested to be the extent of the range of vertebrate host species that contribute significantly to the maintenance of *B, burgdorferi* s.l. spirochetes in nature (*27*). Therefore, cofeeding transmission could also be responsible for *B. afzelii, B. garinii,* and *B. burgdorferi* s.s. infection in larvae that fed on lizards collected in our study, even though skin biopsy results yielded mostly *B. lusitaniae*. Another possible explanation for the presence of non–*B. lusitaniae* spirochetes is that these larvae may have been infected transovarially (*28*). Cofeeding transmission might explain why individual lizards with borreliae negative skin biopsy specimens carry borreliae-positive larvae. Because the quantity of borreliae is low in the vertebrate host and may lodge in deeper organs, detecting them in skin biopsy specimens may not always be possible (*29*). Thus, a negative skin biopsy result does not prove conclusively that the lizard is not infected.

Despite the fact that male lizards hosted >75% of all host-feeding ticks, as well as 79.2% of all infected ticks, the specific infectivity and host-to-tick transmission coefficient were almost the same for male and female lizards. The seasonal activity of green lizards and different patterns in male and female behavior were monitored in the Slovak Karst (I. Majlath, unpub. data). Larger numbers of ticks feeding on male lizards are associated with higher male activity in spring months, when tick activity peaks as well. Male lizards end hibernation first and are active when the air temperature reaches 10°C–12°C. They need to restock the energy that was depleted during winter and to gain energy for fighting other male lizards to compete for territory and females, for seeking female lizards, and for mating. Female activity increases in summer months when they are incubating eggs.

As determined by PCR, the overall prevalence of infection in our sample of questing ticks (19.3%) is consistent with 20.5% found in southern Czech Republic (*20*) but lower than that reported for a geographically close area in western Slovakia (40%–49%) (*30*). The total prevalence was higher in adults (22.5%) than in nymphs (19.6%), which is in agreement with the general pattern of increasing *Borrelia* prevalence through the life stages of ticks as their adults feed on a multiple hosts (*31*). The total prevalence of borreliae in male and female ticks was identical, but the distribution of genospecies was different. *B. garinii* was the predominant genospecies in this locality. *B. garinii* and *B. valaisiana* are the most commonly reported species in central Europe (*32*).

The high prevalence of *B. lusitaniae* in borrelia-positive larvae and nymphs as well as skin biopsy specimens from lizards suggests that green lizards are susceptible and transmission competent for *B. lusitaniae.* On the other hand, a lack or low prevalence of other genospecies in ticks that had fed on lizards may suggest that these genospecies could be negatively selected against by green lizards. A similar suppressive effect of Madeiran wall lizard (*Podarcis dugesii*) on the transmission of spirochetes was observed (*33*). Borreliacidal activity against *B. burgdorferi* s.s. was observed in the lizards *S. occidentalis* and *E. multicarinata* in North America (*15*,*16*). These findings add to the growing support for the hypothesis that there are *Borrelia* species-specific associations with specific reservoir host species that result from *Borrelia* species–specific interactions with host serum complement (*29*).

Significant differences were found in *B. lusitaniae* prevalence in fed larvae compared with questing nymphs ($p \leq 0.001$, df = 1); none of 183 examined nymphs was infected by this genospecies. This finding raises the questions of whether borreliae are eliminated during molting and thus do not contribute to the transmission cycle or whether we were just unable to detect it. Significant differences were found in *B. lusitaniae* prevalence also in fed nymphs compared with questing adults ($p \leq 0.01$, df = 1). The infection prevalence decreased from 74.5% in fed nymphs to 5% in questing adults. Reduction of infection prevalence has been observed in *B. afzelii* from 47% in nymphs engorged on the rodents to 7% in questing nymphs (*9*).

The occurrence of *B. lusitaniae* in ticks is frequent in some areas of the Iberian Peninsula and North Africa, where the organism often represents the only species of *B. burgdorferi* s.l. complex (*13*,*34*). In the rest of the Europe, it has been isolated or detected less frequently, with low prevalence in ticks (*30*,*35*,*36*). The prevalence of *B. lusitaniae* is the highest in southern Europe and can be exported to other areas by hosts such as birds (*37*). The 5S-23S rDNA and flagellin sequences of *B. lusitaniae*–positive ticks and skin biopsy specimens in our study were 100% identical to the *B. lusitaniae* strain Tr213 from a tick in Turkey (*38*). The distribution of this borrelial species may be associated with the distribution range of reservoir hosts, including lizards, that inhabit drier and warmer areas. These ecosystems are less abundant in central Europe than in the Mediterranean. Thus, lizards may influence the transmission cycle of borreliae in some localities in which they are the predominant host for ticks. In our study, we found *B. lusitaniae* in skin biopsy specimens and ticks that fed on green lizards. These findings implicate this species of lizard in the transmission cycle of *B. lusitaniae*. The competence of other lizard species that feed ticks should be also investigated. The low prevalence of *B. lusitaniae* in questing ticks, however, indicates that the ecology of

B. lusitaniae in endemic foci of central Europe is more complex. Further studies that analyze the circulation of *B. burgdorferi* s.l. among a broader spectrum of host species should be undertaken.

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References

1. Wang G, van Dam AP, Schwartz I, Dankert J. Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. Clin Microbiol Rev. 1999;12:633–53.
2. Masuzawa T, Takada N, Kudeken M, Fukui T, Yano Y, Ishiguro F, et al. *Borrelia sinica* sp. nov., a Lyme disease–related *Borrelia* species isolated in China. Int J Syst Evol Microbiol. 2001;51:1817–24.
3. Richter D, Schlee DB, Allgower R, Matuschka FR. Relationships of a novel Lyme disease spirochete, *Borrelia spielmani* sp. nov., with its hosts in Central Europe. Appl Environ Microbiol. 2004;70:6414–9.
4. van Dam AP, Kuiper H, Vos K, Widjojokusumo A, de Jongh BM, Spanjaard L, et al. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. Clin Infect Dis. 1993;17:708–17.
5. Wang G, van Dam AP, Dankert J. Phenotypic and genetic characterization of a novel *Borrelia burgdorferi* sensu lato isolate from a patient with Lyme borreliosis. J Clin Microbiol. 1999;37:3025–8.
6. Foldvari G, Farkas R, Lakos A. *Borrelia spielmanii* erythema migrans, Hungary. Emerg Infect Dis. 2005;11:1794–5.
7. Collares-Pereira M, Couceiro S, Franca I, Kurtenbach K, Schafer SM, Vitorino L, et al. First isolation of *Borrelia lusitaniae* from a human patient. J Clin Microbiol. 2004;42:1316–8.
8. Diza E, Papa A, Vezyri E, Tsounis S, Milonas I, Antoniadis A. *Borrelia valaisiana* in cerebrospinal fluid. Emerg Infect Dis. 2004;10:1692–3.
9. Hanincova K, Schafer SM, Etti S, Sewell HS, Taragelova V, Ziak D, et al. Association of *Borrelia afzelii* with rodents in Europe. Parasitology. 2003;126:11–20.
10. Hanincova K, Taragelova V, Koci J, Schafer SM, Hails R, Ullmann AJ, et al. Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. Appl Environ Microbiol. 2003;69:2825–30.
11. Majlath I, Majlathova V. Green lizard (*Lacerta viridis*) as host for ectoparasites. Proceedings of abstracts and papers of the 5th International Conference on Ticks and Tick-Borne Pathogens. Neuchatel, Switzerland; 2005 29 Aug–2 Sep. Abstract 24.
12. Tomassone L, Bertolotti L, Tramuta C, Nebbia P, Amore G, Ambrogi C, et al. Bacterial tick-borne pathogens in ticks and vertebrate hosts in Tuscany (Italy). Proceedings of abstracts and papers of the 5th International Conference on Ticks and Tick-Borne Pathogens.Neuchatel, Switzerland; 2005 29 Aug–2 Sep.:220–2.
13. Younsi H, Postic D, Baranton G, Bouattour A. High prevalence of *Borrelia lusitaniae* in *Ixodes ricinus* ticks in Tunisia. Eur J Epidemiol. 2001;17:53–6.
14. Clark K, Hendricks A, Burge D. Molecular identification and analysis of *Borrelia burgdorferi* sensu lato in lizards in the southeastern United States. Appl Environ Microbiol. 2005;71:2616–25.
15. Wright SA, Lane RS, Clover JR. Infestation of the southern alligator lizard (*Squamata: Anguidae*) by *Ixodes pacificus* (Acari: Ixodidae) and its susceptibility to *Borrelia burgdorferi.* J Med Entomol. 1998;35:1044–9.
16. Lane RS, Loye JE. Lyme disease in California: interrelationship of *Ixodes pacificus* (Acari: Ixodidae), the western fence lizard (*Sceloporus occidentalis*), and *Borrelia burgdorferi*. J Med Entomol. 1989;26:272–8.
17. Levin M, Levine JF, Yang S, Howard P, Apperson CS. Reservoir competence of the southeastern five-lined skink (*Eumeces inexpectatus*) and the green anole (*Anolis carolinensis*) for *Borrelia burgdorferi*. Am J Trop Med Hyg. 1996;54:92–7.
18. Guy EC, Stanek G. Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. J Clin Pathol. 1991;44:610–1.
19. Black WC, Roehrdanz RL. Mitochondrial gene order is not conserved in arthropods: prostriate and metastriate tick mitochondrial genomes. Mol Biol Evol. 1998;15:1772–85.
20. Derdakova M, Beati L, Pet'ko B, Stanko M, Fish D. Genetic variability within *Borrelia burgdorferi* sensu lato genospecies established by PCR-single-strand conformation polymorphism analysis of the rrfA-rrlB intergenic spacer in *Ixodes ricinus* ticks from the Czech Republic. Appl Environ Microbiol. 2003;69:509–16.
21. Fukunaga M, Hamase A, Okada K, Nakao M. *Borrelia tanukii* sp. nov. and *Borrelia turdae* sp. nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted PCR analysis. Microbiol Immunol. 1996;40:877–81.
22. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25:3389–402.
23. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80.
24. Mather TN, Wilson ML, Moore SI, Ribeiro JM, Spielman A. Comparing the relative potential of rodents as reservoirs of the Lyme disease spirochete (*Borrelia burgdorferi*). Am J Epidemiol. 1989;130:143–50.
25. Gern L, Rais O. Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). J Med Entomol. 1996;33:189–92.
26. Ogden NH, Nuttall PA, Randolph SE. Natural Lyme disease cycles maintained via sheep by co-feeding ticks. Parasitology. 1997;115: 591–9
27. Randolph SE, Gern L, Nuttall PA. Co-feeding ticks: epidemiological significance for tick-borne pathogen transmission. Parasitol Today. 1996;12:472–9.
28. Piesman J, Donahue JG, Mather TN, Spielman A. Transovarially acquired Lyme disease spirochetes (*Borrelia burgdorferi*) in field-collected larval *Ixodes dammini* (Acari: Ixodidae). J Med Entomol. 1986;23:219.
29. Kurtenbach K, De Michelis S, Etti S, Schafer SM, Sewell HS, Brade V, et al. Host association of *Borrelia burgdorferi* sensu lato-the key role of host complement. Trends Microbiol. 2002;10:74–9.
30. Gern L, Hu CM, Kocianova E, Vyrostekova V, Rehacek J. Genetic diversity of *Borrelia burgdorferi* sensu lato isolates obtained from *Ixodes ricinus* ticks collected in Slovakia. Eur J Epidemiol. 1999;15:665–9.
31. Hubalek Z, Halouzka J. Prevalence rates of *Borrelia burgdorferi* sensu lato in host-seeking *Ixodes ricinus* ticks in Europe. Parasitol Res. 1998;84:167–72.

32. Hubalek Z, Halouzka J. Distribution of *Borrelia burgdorferi* sensu lato genomic groups in Europe, a review. Eur J Epidemiol. 1997;13:951–7.
33. Matuschka FR, Eiffert H, Ohlenbusch A, Richter D, Schein E, Spielman A. Transmission of the agent of Lyme disease on a subtropical island. Trop Med Parasitol. 1994;45:39–44.
34. De Michelis S, Sewell HS, Collares-Pereira M, Santos-Reis M, Schouls LM, Benes V, et al. Genetic diversity of *Borrelia burgdorferi* sensu lato in ticks from mainland Portugal. J Clin Microbiol. 2000;38:2128–33.
35. Le Fleche A, Postic D, Girardet K, Peter O, Baranton G. Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int J Syst Bacteriol. 1997;47:921–5.
36. Wodecka B, Skotarczak B. First isolation of *Borrelia lusitaniae* DNA from *Ixodes ricinus* ticks in Poland. Scand J Infect Dis. 2005;37:27–34.
37. Poupon M-A, Lommano E, Humair PF, Douet V, Rais O, Schaad M, et al. Prevalence of *Borrelia burgdorferi* sensu lato in ticks collected from migratory birds in Switzerland. Appl Environ Microbiol. 2006;72:976–9.
38. Guner ES, Hashimoto N, Takada N, Kaneda K, Imai Y, Masuzawa T. First isolation and characterization of *Borrelia burgdorferi* sensu lato strains from *Ixodes ricinus* ticks in Turkey. J Med Microbiol. 2003;52:807–13.

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Transmission of Human and Macaque *Plasmodium* spp. to Ex-Captive Orangutans in Kalimantan, Indonesia

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Data are lacking on the specific diseases to which great apes are susceptible and the transmission dynamics and overall impact of these diseases. We examined the prevalence of *Plasmodium* spp. infections in semicaptive orangutans housed at the Orangutan Care Center and Quarantine, Central Kalimantan, Indonesia, by using a combination of microscopic and DNA molecular techniques to identify the *Plasmodium* spp. in each animal. Previous studies indicated 2 orangutan-specific *Plasmodium* spp., but our data show 4 *Plasmodium* spp. These findings provide evidence for *P. vivax* transmission between humans and orangutans and for *P. cynomolgi* transmission between macaques and orangutans. These data have potential implications for the conservation of orangutans and also for the bidirectional transmission of parasites between orangutans and humans visiting or living in the region.

The following great apes are classified as endangered: the robust chimpanzees, *Pan troglodytes*; the gracile chimpanzee/bonobo, *Pan paniscus*; the gorilla, *Gorilla gorilla*; and the orangutans, *Pongo pygmaeus* and *P. abelii.* Habitat loss and hunting by humans are 2 direct threats to the survival of the great apes (*1*). Until recently, diseases have been overlooked as key threats to primate conservation efforts (*2*). However, recent research has emphasized the threat of disease transmission between human and nonhuman primates and the effects of these diseases on nonhuman primates (*3*). Malaria, caused by protozoan parasites in the genus *Plasmodium*, is 1 disease identified as a potential threat to the conservation of orangutans (*4*). Two species of *Plasmodium* naturally infect orangutans: *P. pitheci*, first isolated from the blood of a Bornean orangutan (*5*), and *P. sylvaticum*, identified from orangutans housed at the Sepilok Orangutan Rehabilitation Centre (SORC), Sabah, Malaysia (*6,7*). Both *P. pitheci* and *P. silvaticum* have tertian periodicities (*5,7*) and are distinguishable from human plasmodia (*7*). Three major studies of orangutan malaria at SORC (*4,5,7*) found prevalences of infection of >50%, which may have been influenced by the unusually high population density of orangutans at SORC, estimated at 100/km^2 (*4*). Wolfe et al. (*4*) found the highest *Plasmodium* spp. prevalence at 93.5% (29/31) in captive animals but 11.6% (5/43) in wild orangutans.

Recent reports indicate that nonhuman primate plasmodia are the source of zoonotic disease outbreaks among humans in Thailand and Malaysia (*8,9*). Although this finding has implications for human disease outbreaks, few studies have investigated the distribution and transmission of *Plasmodium* spp. among orangutans and whether these great apes serve as reservoirs for human infections. Similarly, no studies have indicated that human plasmodia might infect and cause the death of captive or feral orangutans, a finding which would have serious implications for great ape conservation efforts. We report here the identification of plasmodia found in semicaptive and recently

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arrived orangutans at the Orangutan Care Center and Quarantine (OCC&Q) in the province of Central Kalimantan, Indonesia.

Materials and Methods

Study Site and Population

The OCC&Q is located in the village of Pasir Panjang, ≈5 km from the city of Pangkalan Bun in the province of Central Kalimantan, Indonesia. The OCC&Q was established by the Orangutan Foundation International in 1998 to serve as a hospital, orphanage, and rehabilitation center for sick and injured orangutans. Most orangutans are delivered by police or forestry officials after they have been confiscated from illegal pet owners; thus, the history of these animals is often unknown. All orangutans living at the OCC&Q are considered to be semicaptive; during the day, these orangutans have access to a nursery forest where they can learn the skills necessary for survival in the wild. At night these orangutans return to cages for sleep. Approximately 200 orangutans were housed at the OCC&Q during the 2003 study period. Samples were obtained from resident orangutans at OCC&Q as part of biannual health checks done by OCC&Q veterinary staff, and samples were obtained from newly confiscated animals as part of routine medical examinations. Orangutans were grouped into 4 categories on the basis of their residence history at OCC&Q: 1) OCC&Q residents (living at OCC&Q for ≥4 months) (n = 69), 2) newly confiscated arrivals (living at OCC&Q for <4 months) (n = 14), 3) newly confiscated arrivals with a previous history of treatment for malaria (n = 1), 4) newly arrived animals that had at 1 time been ex-captives, previously released back into the forest (n = 2). All animals also were grouped by size, which was used to estimate age: small (<15 kg), medium (15–30 kg), and large (>30 kg) (Table).

Sample Collection and Preservation

Blood samples were collected by an OCC&Q veterinarian with a 25-gauge × $1^5/_8$-inch PrecisionGlide needle (Becton, Dickinson, and Company, Oakville, Ontario, Canada) and a 3-mL syringe. Some animals were sampled more than once so the effectiveness of antimalarial chemotherapy could be monitored. Thin and thick blood smears were prepared, fixed with methanol, stained with 10% Giemsa stain for 30 min, and destained with water (*10*). Samples were examined microscopically under 1,000× magnification, and levels of parasitemia were estimated (*10*). Aliquots of blood (5–10 μL) were placed by pipette onto each of 4 circular areas on Whatman FTA Classic Cards (Whatman Inc., Florham Park, NJ, USA), dried overnight at room temperature, and transported to Simon Fraser University for subsequent analysis.

DNA Extraction

DNA was extracted from the Whatman FTA Classic Cards following the manufacturer's instructions (*11*,*12*). In subsequent PCR analyses, we used an entire punch or 2 μL of eluted DNA per reaction.

PCR Analysis

We used primers designed against the 18S small subunit ribosomal RNA (*4*,*13*). The DNA samples were used in a 3-step PCR process. In step 1, PCR of DNA on the disks or from elutant was amplified with primers rPLU1 (5′-TCA AAG ATT AAG CCA TGC AAG TGA-3′) and rPLU5 (5′-CCT GTT GTT GCC TTA AAC TCC-3′) in a standard 50-μL PCR with a PTC-200 Thermocycler (MJ

Table. Demographic data and infection status of orangutans housed at the Orangutan Care Center and Quarantine (OCC&Q), Central Kalimantan, Indonesia

			Sex		Blood smear positive for *Plasmodium* spp.		DNA positive for *Plasmodium* spp.	
Demographic category	Size*	No.	M	F	M	F	M	F
OCC&Q residents	Small	4	3	1	1	0	0	0
	Medium	61	28	33	7	6	3	1
	Large	4	3	1	0	0	0	0
Newly confiscated arrivals	Small	14	5	9	2	6	2	5
	Medium	0	0	0	0	0	0	0
	Large	0	0	0	0	0	0	0
Newly confiscated arrivals treated previously for malaria	Small	1	0	1	0	0	0	0
	Medium	0	0	0	0	0	0	0
	Large	0	0	0	0	0	0	0
Newly recaptured feral animals	Small	0	0	0	0	0	0	0
	Medium	2	2	0	2	0	2	0
	Large	0	0	0	0	0	0	0
Total	Small	19	8	11	3	6	2	5
	Medium	63	28	33	9	6	5	1
	Large	4	3	1	0	0	0	0

*Size classes were used as an estimate of age. Small orangutans weigh <15 kg, medium orangutans weigh 15–30 kg, and large orangutans weigh >30 kg.

Research; Waltham, MA, USA) under the conditions of 94°C for 4 min, and 35 cycles at 94°C (30 sec), 55°C (1 min), 72°C (1 min), with an additional extension at 72°C (4 min).

In step 2, this product was used in a nested PCR with primers rPLU 3 (5′-TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT-3′) and rPLU Cal 2 (5′-CGC TAT TGG AGC TGG AAT TAC C-3′) in a 25-μL reaction under the conditions of 94°C (4 min) and 35 cycles at 94°C (10 sec), 60°C (10 sec), 72°C (45 sec), with an additional extension at 72°C (4 min). PCR products were size fractionated by electrophoresis on a 1% agarose gel containing ethidium bromide and examined on a BioDoc gel documentation System (UVP, Upland, CA, USA). A ≈500-bp band confirmed *Plasmodium* spp. DNA in the initial PCR sample.

We used the DNA from step 1 that tested positive in step 2 in a third PCR with primers rPLU 3 (5′-TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT-3′) and rPLU Cal (5′-ACA CAW RGT KCC TCT AAG AAG C-3′) by using BD Sprint Advantage Single Shots (Clontech, Palo Alto, CA, USA) under the conditions of 95°C (1 min), and 35 cycles at 95°C (30 sec), and 58° (3 min), with an additional extension at 58°C (3 min). These nested primers amplified an ≈1,500-bp fragment that contains 3–4 variable regions of the ribosomal sequence that can be used for species determination. After electrophoresis, PCR products were excised from the gel, purified by using a Qiagen Gel Purification Kit (Qiagen, Valencia, CA, USA), and ligated into pGEM-T-Easy Vector (Promega, Madison WI, USA). Putative transformants were identified by using blue-white screening of XL1-Blue Cells (Stratagene, La Jolla, CA, USA), grown overnight in 5 mL LB medium with ampicillin (100 μg/μL) and purified by using Wizard Plus Miniprep DNA Purification System (Promega). Sequencing of clones was done by using BigDye Chemistry (version 3.1) (Applied Biosystems, Foster City, CA, USA) and with the plasmid primers SP6 and T7 (*14*).

Data Analysis

The nucleotide sequences obtained were compared with those *Plasmodium* spp. sequences available in public databases by using BLASTN (nucleotide-nucleotide) (available from www.ncbi.nlm.nih.gov/blast/). All available sequences of the *Plasmodium* spp. 18S sRNA gene, which contained complete sequences of our target region, were downloaded.

Results

Field Results

We collected 97 blood samples from 86 animals: 19 small, 63 medium, and 4 large orangutans, of which 41 were males and 45 were females (Table). Of the 69 OCC&Q residents tested (34 males, 35 females), 14 (20.3%) tested positive for *Plasmodium* spp. infections (8 males and 6 females) (Table). Eight of 14 newly arrived animals tested positive for *Plasmodium* spp. (57%) (2 small males and 6 small females) (Table). The newly arrived animal treated previously for malaria symptoms proved negative for *Plasmodium* spp. Both previously released ex-captive, medium-sized male orangutans, brought to OCC&Q from a release site in Tanjung Puting National Park for treatment of amebic dysentery, tested positive for *Plasmodium* spp. (Table).

DNA Analysis

We amplified, cloned, and sequenced a ≈1,500-bp segment of the *Plasmodium* spp. 18S sRNA gene from 13 of the 24 orangutans whose blood had been found positive for *Plasmodium* spp.: 4 from OCC&Q residents (3 males, 1 female), 7 from newly confiscated arrivals (2 males, 5 females), and 2 newly arrived feral animals (2 males) (Table). We aligned the sequences with similar sequences available in the databases, and generated phylogenetic trees based on a nearest neighbor analysis at the nucleotide level. We then aligned at the nucleotide level the sequences we obtained from our 13 *Plasmodium* spp.–infected orangutans. Phylogenetic trees showing the nearest neighbor relationships of our sequences were created from these alignments (Figure 1).

The 13 sequences we obtained form 4 distinct groups at the nucleotide level. On the basis of these groupings, we designated samples VS25, VS28, VM75, and VM82 as group 1; samples VS14 and VM88 as group 2; VS21, VS32, VS33, VM40, VM55, and VM71 as group 3; and VS63 as group 4. From each of these groupings, we selected 1 sequence to represent the entire group. These representative samples were then aligned at the nucleotide and translated amino acid level with available sequences downloaded from the databases to generate a phylogenetic tree (Figure 2).

Group 1 (representative sequence VM82) consists of 1,519 bp at the nucleotide level and translates to a putative protein with a length of 506 amino acids (aa). VM82 shares the greatest sequence identity with *P. cynomolgi* (94%) and *P. inui* (95%). A phylogenetic tree shows the close association with *P. inui* and *P. cynomolgi*. We then aligned VM82 solely with its 2 closest homologs, *P. inui* and *P. cynomolgi.* These 3 sequences are highly conserved but have variable areas between bases 106–155, 633–672, 720–730, and 1,018–1,050, which indicate a closer similarity with *P. cynomolgi*. Because of differences between VM82 and *P. cynomolgi* in the region of bases 143–155, 646–673, and 1,023–1,043, we designated this group as a *P. cynomolgi*–like parasite.

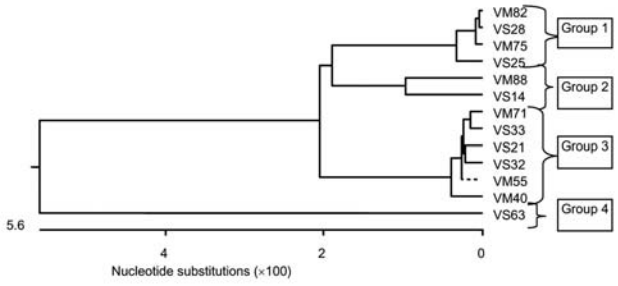

Figure 1. Phylogenetic tree of obtained sequences at the nucleotide level. Phylogenetic analysis of 14 individual sequences of the 18S small subunit ribosomal RNA gene (nucleotide level) isolated from the blood of orangutans housed at the Orangutan Care Center and Quarantine by PCR. Group 1 (*Plasmodium cynomolgi*–like) is represented by sequences from VS25, VS28, VM75, and VM82; group 2 (*P. inui*–like) is represented by sequences from VS14 and VM88; group 3 (*P. cynomolgi*) is represented by VS21, VS32, VS33, VM40, VM55, and VM71; and group 4 (*P. vivax*) is represented by sequence VS63.

Group 2 (representative sequence VM88) consists of 1,544 bp at the nucleotide level and translates to a putative protein with a length of 514 aa. Throughout the variable regions, VM88 shares substantial similarity with *P. hylobati* (89%–96%), *P. inui* (90%–97%), *P. fieldi*, (89%–95%) and *P. cynomolgi* (90%–95%). We then aligned VM88 solely with its 2 closest homologs, *P. inui* and *P. cynomolgi*. These 3 sequences are highly conserved but show 2 large variable areas between bases 113–175 and 630–670. Despite sharing the greatest homology with *P. inui*, differences between VM88 and *P. inui* in the region of bases 110–175, 712–735, 789–887, and 1,495–1,551 indicate that this is a *P. inui*–like parasite.

Group 3 (representative sequence VM40) consists of 1,515 bp at the nucleotide level and translates to a putative protein with a length of 505 aa. VM40 shares significant identity with 3 *Plasmodium* spp. at the nucleotide level: *P. hylobati*, (96%–97%), *P. inui* (95%–97%), and *P. cynomolgi* (96%–98%). Alignment of VM40 solely with its 2 closest sequences indicated this sequence is homologous to *P. cynomolgi*.

Group 4, sequence VS63, consists of 1,582 bp at the nucleotide level and translates to a putative protein with a length of 526 aa. At the nucleotide level, VS63 shares the greatest identity with *P. simium* (97%–98%) and *P. vivax* (96%–98%).

Discussion

Our results indicate that newly arrived orangutans to OCC&Q are statistically more likely to be infected with *Plasmodium* spp. than resident orangutans (χ^2 = 8.11, degrees of freedom [df] = 1, p<0.01). Because these animals were confiscated from humans, we do not know their history or often the specific geographic region from which they originated. Increased levels of stress caused by time spent in direct contact with humans, reduced arboreality, and lack of a nutritious and normal diet may contribute to increased levels of infection (*4*).

Small orangutans were significantly more likely to be infected with *Plasmodium* spp. than medium orangutans (χ^2 = 3.91, df = 1, p<0.05) or medium and large orangutans combined (χ^2 = 4.59, df = 1, p<0.05). Whether these results are coincidental or whether older animals have some protection against reinfections with *Plasmodium* spp. has not been studied. In humans, most deaths attributed to malaria occur in young children, and evidence of age-acquired immunity against *Plasmodium* spp. has been found (*15*). However, such studies in other primates are few, and we can only speculate that the same phenomenon might be evident here.

We were unable to amplify *Plasmodium* spp. DNA from 9 of the orangutans identified in the field as positive. This may have been due to poor blood preservation, DNA degradation, infections with very low levels of parasitemia, or a misidentification of *Plasmodium* spp. in the initial blood smears.

No published data are available on the level of illness and death suffered by orangutans as a direct result of malaria, nor do data exist on the ability of the orangutan immune response to clear these infections. As a result, the orangutan plasmodia have been considered benign. However, orangutan rehabilitation facilities in Sumatra and Kalimantan have reported the elimination of debilitating,

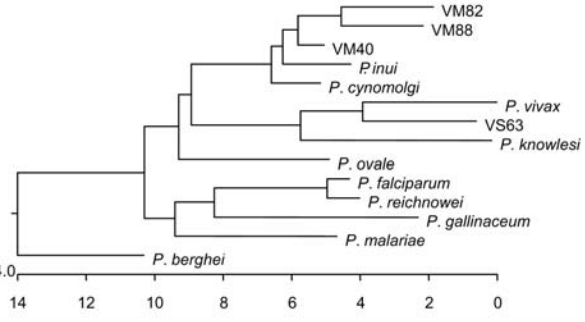

Figure 2. Phylogenetic tree of small subunit ribosomal RNA from different *Plasmodium* spp. Sequences were downloaded from GenBank, aligned by using CLUSTAL W (Megalign, DNA Star, Madison, WI, USA) and the tree generated by nearest-neighbor analysis. Once the sequences were aligned, we also aligned our representative sequences with the 2 nearest matches for more detailed determination of closest associations. Sequences used and their GenBank accession nos. were *P. gallinaceum* (M61723), *P. berghei (*AJ243513), *P. falciparum* (AL929354), *P. ovale* (AJ001527), *P. malariae* (AF88000), *P. vivax* (U03080), *P. cynomolgi* (L08241), *P. fragile* (M61722), *P. knowlesi* (U83876), *P. reichenowi* (Z25819), *P. simium* (U69605), and *P. inui* (U72541), clone 40 (*P. cynomolgi* [DQ660816]), clone 63 (*P. vivax* [DQ660817]), clone 82 (*P. cynomolgi*–like [DQ660818]), and clone 88 (*P. inui*–like [DQ660819]).

malarialike symptoms after treatment with antimalarial drugs ([*16*]; C. van Schaik, pers. comm., 2002). In 2002, OCC&Q treated malarialike symptoms in several orangutans with antimalarial drugs. These animals responded to treatment and returned to normal activity, but this does not definitively prove that their illnesses were the result of malaria (*16*). Because of the paucity of studies on malaria in orangutans, all done at SORC (*4*,*7*), we can only compare our data gathered at OCC&Q with data on the semicaptive orangutans tested at SORC (*4*). However, that study found a high prevalence of infection (93.5%) compared with our study (20.3%). Wolfe et al. (*4*) suggest that several behavioral and ecologic factors may contribute to higher rates of infection with *Plasmodium* spp. among semicaptive orangutans such as decreased arboreality, decreased day ranges, changes in social structure, increased population density, dietary changes, and stress. Each of these factors is also found to some degree in the orangutans housed at OCC&Q. Many ecologic factors differ between these 2 study sites. The region around the OCC&Q is primarily peat swamp forest (*17*,*18*), which typically does not support the growth and development of mosquito larvae. Wolfe et al. (*4*) also discussed the potential role of human activities that contributed to the high prevalence of *Plasmodium* spp. at SORC. These included the proximity of SORC to human settlements, very high densities of orangutans, and the effects of human-made structures such as drainage ditches, which increase the availability of standing water and vector mosquito population. However, no data exist on what mosquito species inhabit these regions or which species might transmit nonhuman plasmodia to orangutans.

Our DNA analyses show 4 distinct groups of *Plasmodium* spp. in the orangutans housed at OCC&Q, on the basis of an ≈1,500-bp segment of the 18S sRNA, considered an "ideal target" for *Plasmodium* spp. identification (*19*). Previous studies that relied on parasite morphology found 2 plasmodia in orangutans; *P. pitheci* and *P. silvaticum* (*4*,*7*), but no DNA sequences with which we could compare our data are available in the databanks for these 2 species. The macaque parasite *P. inui* has also been reported in orangutans housed at SORC (*4*), but these data have yet to be published. Two of our 4 sequences (groups 1 and 2) may possibly represent *P. silvaticum* and *P. pitheci* sequences. These are members of the *P. vivax*/Southeast Asian primate *Plasmodium* spp. group of parasites as are the macaque plasmodia *P. cynomolgi*, *P. inui*, *P. fragile*, and *P. knowlesi* (*20*–*26*).

The group 3 sequences align best with *P. cynomolgi*, a macaque *Plasmodium* sp. that can infect humans. *Plasmodium cynomolgi*, first identified in Javan *Macaca fascicularis* (*5*), is also a member of the *P. vivax*/Southeast Asian primate plasmodia group (*20*–*26*) and has been studied as the primate counterpart to human *P. vivax* (*27*). The presence of this parasite in orangutans indicates the cross-species transfer of *Plasmodium* spp. between macaques and orangutans. Macaques are common throughout Kalimantan and often can be seen crossing the road into and out of the nursery forest used by orphaned orangutans housed at OCC&Q. Although no confirmed reports exist of orangutans being infected with plasmodia specific to other primates, humans have been infected naturally with at least 2 species of macaque plasmodia (*5*,*8*,*9*). A macaque parasite, *P. knowlesi*, naturally infects humans in Malaysia (*5*) and continues to be an important zoonotic infection in Southeast Asia (*8*,*9*). Finding 1 of these species in orangutans housed at OCC&Q would not be surprising.

The group 4 sequence is the most relevant. This sequence aligns most closely with *P. simium* (found only in the New World) and *P. vivax* (found throughout Southeast Asia) and secondarily with *P. knowlesi* (Figure 2). When we compared several *P. knowlesi* sequences with each other, with VS63, and with *P. vivax*, the latter 2 were essentially identical, and BLAST pairwise comparison generated 1 contiguous sequence with >98% shared identity. Areas of notable differences were found between VS63 and *P. knowlesi*. The BLAST pairwise comparison indicates 5 discrete regions of alignment separated by nonsimilar regions, which indicates that VS63 is not a known variant of *P. knowlesi*.

P. vivax and *P. simium* are genetically indistinguishable at 13 microsatellite loci and 8 tandem repeats (*27*). Because *P. simium* does not exist in Southeast Asia, we have the first report of an orangutan being infected with human *P. vivax*. This orangutan was a recent arrival at OCC&Q, had been confiscated from human captivity, had extensive interactions with humans in a domestic setting, and had arrived at OCC&Q with a low-level infection that was untreated 3 months before this study began, which indicates a fully functional infection.

P. vivax is one of the most widespread of the human plasmodia; it infects 70–80 million persons in the low-lying, coastal, and marshy regions of the world (*5*,*24*). Data on the human plasmodia of Central Kalimantan are not easily accessible through the available scientific databases. We assume that *P. vivax* is present in this region on the basis of the results of our study, the widespread nature of this parasite (*5*,*24*), and reports of chloroquine-resistant *P. vivax* in neighboring West Kalimantan (*28*). That *P. vivax* is infective to orangutans is not surprising. Current evidence suggests that *P. vivax* originated 80,000–10,000 years ago from a macaque *Plasmodium* sp. (*22*–*24*). Because humans are genetically closer to orangutans than to macaques, if *P. vivax* arose as the result of a recent host switch, then orangutans also could be infected with *P. vivax*.

Our data indicating that orangutans can be infected with human *P. vivax*, and the corresponding infection of humans with macaque plasmodia (*8*,*9*) emphasize the potential importance of the bidirectional transmission of these parasites between humans and nonhuman primates living in close proximity. Increasing our understanding of potential host species and phylogenetic associations of closely related parasites may help identify the origins of human diseases (*3*,*29*,*30*).

The data presented here suggest that Bornean orangutans (*Pongo pygmaeus*) may be infected by 4 species of plasmodia; 2 of these may represent the previously identified orangutan plasmodia *P. pitheci* and *P. silvaticum*. Macaque malaria in orangutans suggests cross-species transmission of a parasite between macaques and orangutans living sympatrically in Kalimantan (as had been described for human infections with macaque malaria in Thailand and Malaysia) (*8*,*9*). Orangutans also may be susceptible and may be exposed to infection from the human parasite, *P. vivax*, although few data are available on the symptoms of macaque or human malaria infections in orangutans. Nonetheless, these findings could have important implications for orangutan conservation and rehabilitation programs and for humans who live in close proximity to orangutans. The role of humans and great apes as reservoirs of parasites that can be shared and transmitted between both hosts has not been well-studied. Conservation and rehabilitation programs that permit visits by humans must take into consideration the exchange of parasites between humans and endangered species, the implications of human parasites on the survival of the great apes in these centers, and the potential of these animals to serve as reservoirs of human parasites.

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References

1. Butynski TM. Africa's great apes. In: Beck BB, Stoinski TS, Hutchins M, Maple TL, Norton B, Rowan A, et al., editors. Great apes and humans: the ethics of coexistence. Washington: Smithsonian; 2001. p. 3–56.
2. Wallis J, Lee DR. Primate conservation: the prevention of disease transmission. Int J Primatol. 1999;20:803–26.
3. Jones-Engel L, Schillaci MA, Engel G, Paputungan U, Froehlich JW. Characterizing primate pet ownership in Sulawesi: implications for disease transmission. In: Paterson JD, Wallis J, editors. Commensalism and conflict: the human primate interface. Special topics in primatology, vol. 4. Norman (OK): American Society of Primatologists; 2005. p. 97–221.
4. Wolfe ND, Karesh WB, Kilbourne AM, Cox-Singh J, Bosi EJ, Rahman HA, et al. The impact of ecological conditions on the prevalence of malaria among orangutans. Vector Borne Zoonotic Dis. 2002;2:97–103.
5. Coatney GR, Collins WE, Warren M, Contacos PG. The primate malarias. Washington: National Institute of Allergy and Infectious Disease; 1971.
6. Garnham PCC, Rajapaksa N, Peters W, Kilick-Kendrick R. Malaria parasites of the orang-utan *(Pongo pygmaeus).* Ann Trop Med Parasitol. 1972; 66:284–94.
7. Peters W, Garnham PCC, Killick-Kendrick R, Rajapaska N, Cheong WH, Cadigan FC. Malaria of the orang-utan *(Pongo pygmaeus)* in Borneo. Philos Trans R Soc Lond B Biol Sci. 1976;275:439–82.
8. Jongwutiwes S, Putaportip C, Iwasaki T, Sata T, Kanbara H. Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. Emerg Infect Dis. 2004;10:2211–3.
9. Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet. 2004;363:1017–24.
10. Cheesbrough M. District laboratory practice in tropical countries, part 1. Cambridge (UK): Cambridge University Press; 1988.
11. Whatman International, Ltd. FTA protocols. 2000; 1–36 [cited 2006 Oct 4]. Available from http://www.whatman.com
12. Whatman International, Ltd. Eluting genomic DNA from FTA cards using room temperature pH treatment. Whatman application note; 2004 [cited 2006 Oct 4]. Available from http://www.whatman.com
13. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999;60:687–92.
14. Lowenberger C, Charlet M, Vizioli J, Kamal S, Richman A, Christensen BM, et al. Antimicrobial activity spectrum, cDNA cloning, and mRNA expression of a newly isolated member of the Cecropin family from the mosquito vector *Aedes aegypti*. J Biol Chem. 1999;274:20092–7.
15. Hviid L. Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. Acta Trop. 2005;95:270–5.
16. Garriga RM. Andriansyah H. Annual health report 2002: Orangutan Care Center & Quarantine, Pasir Panjang, Kalimantan Tengah, Indonesia. Pasir Panjang (Indonesia): Orangutan Care Center & Quarantine; 2002.
17. Galdikas BMF. Orangutan diet, range and activity at Tanjung Puting, Central Borneo. Int J Primatol. 1988;9:1–35.
18. MacKinnon K, Hatta G, Halim H, Mangalik A. Ecology of Kalimantan: Indonesian Borneo. Ecology of Indonesia series, vol. III. Singapore: Periplus Editions; 1996.
19. Li J, Wirtz RA, Mcconkey GA, Sattabongkot J, Waters AP, Rogers MJ, et al. *Plasmodium:* genus-conserved primers for species identification and quantitation. Exp Parasitol. 1995;81:182–90.
20. Escalante AA, Ayala FJ. Evolutionary origin of *Plasmodium* and other Apicomplexa based on rRNA genes. Proc Natl Acad Sci U S A. 1995;92:5793–7.

21. Escalante AA, Freeland DE, Collins WE, Lal AA. The evolution of primate malaria parasites based on the gene encoding cytochrome b from the linear mitochondrial genome. Proc Natl Acad Sci U S A. 1998;95:8124–9.
22. Escalante AA, Cornejo OE, Freeland DE, Poe AC, Durrego E, Collins WE, et al. A monkey's tale: the origin of *Plasmodium viv*ax as a human malaria parasite. Proc Natl Acad Sci U S A. 2005;102:1980–5.
23. Leclerc MC, Hugot JP, Durand P, Renaud F. Evolutionary relationships between 15 *Plasmodium* species from Old and New World primates (including humans): a 18S rDNA cladistic analysis. Parasitology. 2004;129:677–84.
24. Mu J, Joy DA, Duan J, Huang Y, Carlton J, Walker J, et al. Host switch leads to emergence of *Plasmodium vivax* malaria in humans. Mol Biol Evol. 2005;22:1686–93.
25. Rathore D, Wahl AM, Sullivan M, McCutchan TF. A phylogenetic comparison of gene trees constructed from plastid, mitochondrial and genomic DNA of *Plasmodium* species. Mol Biochem Parasitol. 2001;114:89–94.
26. Waters AP, Higgins DG, McCutchan TF. Evolutionary relatedness of some primate models of *Plasmodium*. Mol Biol Evol. 1993;10:914–23.
27. Leclerc MC, Durand P, Gauthier C, Patot S, Billotte N, Menegon M, et al. Meager genetic variability of the human malaria agent *Plasmodium vivax*. Proc Natl Acad Sci U S A. 2004;101:14455–60.
28. Fryauff DJ, Tuti S, Mardi A, Masbar S, Patipelohi R, Leksana B, et al. Chloroquine-resistant *Plasmodium vivax* in transmigration settlements of West Kalimantan, Indonesia. Am J Trop Med Hyg. 1998;59:513–8.
29. Jones-Engel L, Engel GA, Schillaci MA, Rompis A, Putra A, Suaryana KG, et al. Primate-to-human retroviral transmission in Asia. Emerg Infect Dis. 2005;11:1028–35.
30. Wolfe ND, Escalante AA, Karesh WB, Kilbourn A, Spielman A, Lal AA. Wild primate populations in emerging infectious disease research: the missing link? Emerg Infect Dis. 1998;4:149–58.

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Borrelia garinii in Seabird Ticks (*Ixodes uriae*), Atlantic Coast, North America

Robert P. Smith, Jr,* Sabir Bin Muzaffar,† Jennifer Lavers,† Eleanor H. Lacombe,* Bruce K. Cahill,* Charles B. Lubelczyk,* Allen Kinsler,* Amy J. Mathers,* and Peter W. Rand*

Borrelia garinii is the most neurotropic of the genospecies of *B. burgdorferi* sensu lato that cause Lyme disease in Europe, where it is transmitted to avian and mammalian reservoir hosts and to humans by *Ixodes ricinus*. *B. garinii* is also maintained in an enzootic cycle in seabirds by *I. uriae*, a tick found at high latitudes in both the Northern and Southern Hemispheres. To determine whether *B. garinii* is present in seabird ticks on the Atlantic Coast of North America, we examined 261 *I. uriae* ticks by polyclonal antiborrelial fluorescent antibody. Ten of 61 ticks from Gull Island, Newfoundland, were positive for borreliae by this screen. Amplicons of DNA obtained by PCR that targeted the *B. garinii* rrs-rrla intergenic spacer were sequenced and matched to GenBank sequences for *B. garinii.* The potential for introduction of this agent into the North American Lyme disease enzootic is unknown.

In Europe, Lyme disease is caused by 3 genospecies of *Borrelia burgdorferi* (i.e., *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto), while in North America only *B. burgdorferi* sensu stricto, the genospecies present in *I. scapularis* ticks, has been implicated in human disease. *B. garinii*, the most neurotropic of these 3 genospecies, causes most neurologic Lyme disease in Europe, including cases of meningopolyneuritis and, rarely, encephalomyelitis (*1*–*3*). The presence of multiple pathogenic genospecies that cause Lyme disease in Europe complicates serodiagnostic testing (*4*).

In Eurasia, *B. garinii* is transmitted to avian and rodent hosts and to humans by *I. ricinus*, the sheep or forest tick, and *I. persulcatus*, the taiga tick (*5*–*9*). *I. uriae*, the seabird tick, also maintains this agent in a "silent" enzootic cycle in seabirds at their nesting sites over a wide but discontinuous area (*10*–*13*). Although these 2 enzootic cycles are generally geographically and ecologically separate, interchange of *B. garinii* strains may occur at sites where both vectors coexist (*14*). The risk for seabird-associated strain types of *B. garinii* to cause Lyme disease, however, is not known (*15*).

Although *B. garinii* is present in seabird ticks in a nearly circumpolar distribution in both the Northern and Southern Hemispheres (*12*,*13*), including Alaska, the presence of *B. garinii* in *I. uriae* ticks at sites on the North Atlantic Coast has not previously been documented. We sought to determine whether *B. garinii* is present in ticks obtained from colonial seabird nesting sites on the Atlantic Coast of North America.

Methods

Seabird researchers working at several sites on the Atlantic Coast in the United States and Canada (Figure 1) submitted *I. uriae* ticks to our laboratory for analysis (*16*). Ticks were identified to species, stage, and degree of engorgement (*17*). A subset of ticks was dissected, and midguts were screened for spirochetes by fluorescent microscopy by using a polyclonal antiborrelial antibody (*18*).

DNA was extracted from *Borrelia*-positive ticks by using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). DNA amplification was performed in a designated room with genus-specific primers that include the partial sequence of rrs-rrla intergenic spacer region as described by Bunikis et al. (*19*) with use of negative con-

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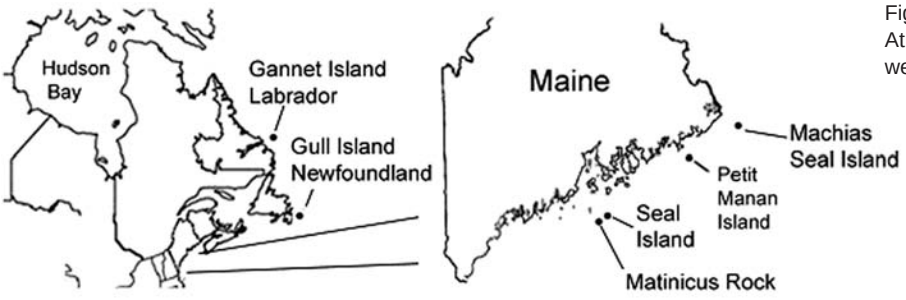

Figure 1. Locations in Maine (USA) and Atlantic Canada where *Ixodes uriae* ticks were collected.

trols. Amplification products were viewed on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. At a second laboratory, ticks positive by fluorescent antibody screen were prepared as above for DNA extraction, and PCR was performed by using primers directed at the 16S ribosomal DNA. Sequences of amplicons obtained at both laboratories were confirmed to be *B. garinii* by comparison with known sequences in the GenBank database.

Results

I. uriae ticks submitted from 6 seabird colony sites and several hosts in the United States and Canada were processed (Table). Through 2005, 880 *I. uriae* ticks recovered primarily from Atlantic puffin chicks or their nests, were submitted from Maine sites, and another 383 were submitted from sites on the Atlantic Coast of Canada. Over 200 ticks from Maine sites and 61 ticks from Canadian sites off the coast of Newfoundland and Labrador were screened for borreliae by fluorescent microscopy.

Spirochetes were detected only in ticks from Gull Island, Newfoundland (47° 15′N, 52° 46′W), where 9 of 22 adults and 1 of 39 nymphal ticks were positive. DNA was extracted from 2 of these ticks, and PCR showed a 1,900-base amplicon of the rrs-rrls intergenic spacer region that matched with *B. garinii* on comparison with GenBank sequences. Two additional ticks were examined in the laboratory of Sam Telford (Tufts University School of Veterinary Medicine, Grafton, MA, USA) by means of PCR targeting of 16S ribosomal DNA and again confirmed a match for *B. garinii* (GenBank bankit no. 800902 DG463373). Figure 2 shows the sequence from one of the ticks shown in an alignment with sequences from *B. burgdorferi* strain B31 and a representative *B. garinii* sample from GenBank.

Discussion

The finding of *B. garinii* in *I. uriae* ticks from Gull Island, Newfoundland, adds to the known distribution of this agent and increases the likelihood that this agent is present in other colonial seabird nesting sites on the Atlantic Coast of North America, as is the case in Europe. The recent emergence of *I. scapularis* in coastal Maine and some Maritime Canadian sites (*20*,*21*) brings these 2 enzootic cycles of different genospecies of *B. burgdorferi* into proximate areas, although their ecologic settings differ. The public health importance of this finding depends on the probability of the introduction of *B. garinii* into emergent *I. scapularis*-vectored *B. burgdorferi* s.s. cycles and its potential maintenance in this cycle. The public health effects also depend on the pathogenic potential for human disease caused by seabird-associated strains *of B. garinii* (*15*).

The remote geographic and, at some sites, isolated topographic location of colonial seabird colonies provide significant barriers to the introduction of *B. garinii* into other vector ticks and their reservoir hosts. *I. scapularis* is

Table. Submissions of *Ixodes uriae* from coastal Maine (USA) and Canada, 1996–2005

	Host species							
Site	Atlantic puffin (*Fratercula arctica*)	Murre (*Uria aalge*)/Razorbill (*Alca torda*)	Black guillemot (*Cepphus grille*)	Herring gull (*Larus argentatus*)	Common eider (*Somateria mollissima*)	On humans	Flag/drag sampling	Soil/litter sample
Machias Seal Island*	218					5	52	
Matinicus Rock*	258	23	25			210		
Petit Manan Island*	56		12					
Seal Island*	46							
Gannet Island†	111	88			8	72	31	
Gull Island‡	22			22		5	76	200

*Maine, USA.
†Labrador, Canada.
‡Newfoundland, Canada.

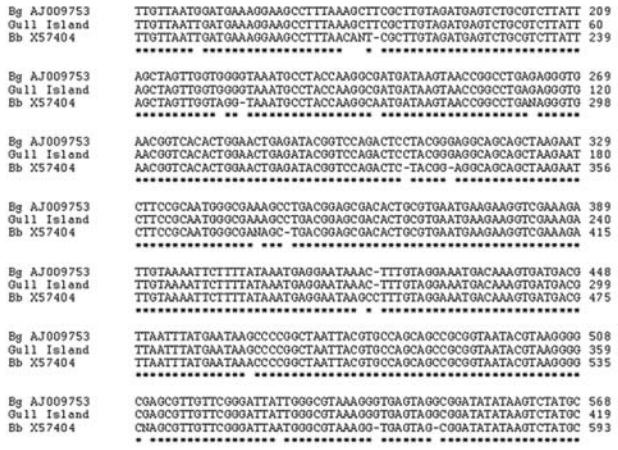

Figure 2. Alignments of 16S RNA sequences from GenBank: Bg AJ009753, *Borrelia garinii*; Bb X57404, *B. burgdorferi* strain B31, Gull Island, Newfoundland, Canada.

dispersed to remote coastal islands of the North American Atlantic Coast during bird migration (*22*,*23*), but its establishment at these sites requires the presence of deer (*24–26*). With rare exception, deer are absent from sites with large seabird colonies, which are usually limited to offshore islands. Dispersal of infected *I. uriae* to proximate coastal areas by seabird hosts is unlikely because most species of seabirds parasitized by *I. uriae* are highly philopatric and forage at sea. One exception might be gulls, which may move between coastal and island sites. Passerine birds, which may forage near seabird colonies, provide another potential mechanism for dispersal of *B. garinii*, either through movement of infected *I. uriae* ticks or by serving as reservoir hosts of this agent. However, the frequency of parasitism of passerine birds by *I. uriae* ticks is unknown.

If *B. garinii* was introduced into *I. scapularis* ticks, its maintenance in this cycle would depend on the vector competence of *I. scapularis* for *B. garinii*, the reservoir competence of available hosts, and perhaps the population genetics and strain diversity of *B. garinii* (*14*,*27*). Although *I. scapularis* is vector-competent for transmission of *B. garinii* to rodents, its efficiency of transmission appears lower than for *B. burgdorferi* s.s. (*28*). In addition, the vector competence of *I. scapularis* for seabird-associated strains of *B. garinii* has not been tested. The presence of similar ribotypes of *B. garinii* in *I. ricinus* ticks on a European island suggests that interchange of different *B. garinii* strains in different ecologic cycles may occur (*14*).

To determine the public health importance of *B. garinii* in seabird colonies along the North Atlantic coast, additional studies on the issues of dispersal, vector competence, and reservoir host competence are needed. All strain types of *B. garinii* may not be pathogenic for humans, and future studies should also address the potential for seabird-associated strains to cause disease.

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Dr Smith is an infectious disease physician and codirector of the Vector-borne Disease Laboratory at the Maine Medical Center Research Institute. His research interests include the ecology of Lyme disease emergence and the strain diversity of *B. burgdorferi*.

References

1. Steere AC, Coburn J, Glickstein L. The emergence of Lyme disease. J Clin Invest. 2004;113:1093–101.
2. Oschmann P, Dorndoff W, Hornig C, Schafer C, Wellensiek HJ, Pflughaupt KW. Stages and syndromes of neuroborreliosis. J Neurol. 1998;245:262–72.
3. Halpern JJ. Nervous system Lyme disease. Vector Borne Zoonotic Dis. 2002;2:241–7.
4. Hauser U, Lehnert G, Lobentamzer R, Wilske B. Interpretation criteria for standardized Western blots for three European species of *Borrelia burgdorferi* sensu lato. J Clin Microbiol. 1997;35:1433–44.
5. Gern L, Humair P-F. Ecology of *Borrelia burgdorferi* sensu lato in Europe. In: Gray JS, Kahl O, Lane RS, Stanek G, editors. Lyme borreliosis: biology, epidemiology, and control. New York: CABI Publishing; 2002. p. 149–74.
6. Nakao M, Miyamoto K, Fukunaga M. Lyme disease spirochetes in Japan: enzootic transmission cycles in birds, rodents, and *Ixodes persulcatus* ticks. J Infect Dis. 1994;170:878–82.
7. Kurtenbach K, Peacey M, Rijpkema SGT, Hoodless AN, Nuttall PA, Randolph SE. Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. Appl Environ Microbiol. 1998;64:1169–73.
8. Humair P-F, Postic D, Wallich R, Gern L. An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. Zentrabl. Bakteriol. 1998;287:521–38.
9. Richter D, Endepols S, Ohlenbusch A, Eiffert H, Spielman A, Matuschka FR. Genospecies diversity of Lyme disease spirochetes in rodent reservoirs. Emerg Infect Dis. 1999;5:291–6.
10. Olsen B, Jaenson TGT, Noppa L, Bunikis J, Bergstrom S. A Lyme borreliosis cycle in seabirds and *Ixodes uriae* ticks. Nature. 1993;362:340–2.
11. Olsen B, Duffy DC, Jaenson TGT, Gylfe A. J. Bonnedahl J, Bergstrom S. Transhemispheric exchange of Lyme disease spirochetes by seabirds. J Clin Microbiol. 1995;33:3270–4.
12. Gylfe A, Olsen B, Strasevicius D, Ras NM, Weihe P, Noppa L, et al. Isolation of Lyme disease borrelia from puffins (*Fratercula arctica*) and seabird ticks (*Ixodes uriae*) on the Faeroe Islands. J Clin Microbiol. 1999;37:890–6.

13. Gylfe A, Yabuki M, Drotz M, Bergstrom S, Fukunaga M, Olsen B. Phylogenetic relationships of *Ixodes uriae* (Acari: Ixodidae) and their significance to transequatorial dispersal of *Borrelia garinii*. Hereditas. 2001;134:195–9.
14. Bunikis J, Olsen B, Fingerle V, Bonnedahl J, Wilske B, Bergstrom S. Molecular polymorphism of the Lyme disease agent *Borrelia garinii* in Northern Europe is influenced by a novel enzootic *Borrelia* focus in the North Atlantic. J Clin Microbiol. 1996;34:364–8.
15. Baranton G, Seinost G, Theodore G, Postic D, Dykhuizen D. Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity. Res Microbiol. 2001;152:149–56.
16. Keirans JE, Lacombe EH. First records of *Amblyomma americanum*, *Ixodes* (*Ixodes*) *dentatus*, and *Ixodes* (*Ceratixodes*) *uriae* (Acari: Ixodidae) from Maine. J Parasitol. 1998;84:629–31.
17. Cooley RA, Kohls GM. The genus *Ixodes* in North America. Nat. Inst Health Bull (Edinb). 1945;184:223–8.
18. Donahue JG, Piesman J, Spielman A. Reservoir competence of white-footed mice for Lyme disease spirochetes. Am J Trop Med Hyg. 1987;36:92–6.
19. Bunikis J, Garpmo U, Tsao J, Berglund J, Fish D, Barbour AG. Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology. 2004;150:1741–55.
20. Bell CR, Specht HB, Coombs BA. The search for *Ixodes dammini* and *Borrelia burgdorferi* in Nova Scotia. Can J Infect Dis. 1992;3:224–30.
21. Scott JD, Fernando K, Banerjee SN, Durden LA, Byrne SK, Banerjee M, et al. Birds disperse ixodid (Acari: Ixodidae) and *Borrelia burgdorferi*–infected ticks in Canada. J Med Entomol. 2001;38:493–500.
22. Smith RP Jr, Rand PW, Lacombe EH, Morris SR, Holmes DW, Caporale DA. Role of bird migration in the long-distance dispersal of *Ixodes dammini*, the vector of Lyme disease. J Infect Dis. 1996;174:221–4.
23. Morshed MG, Scott JD, Banerjee SN, Banerjee M, Fitzgerald T, Fernando K, et al. First isolation of Lyme disease spirochete, *Borrelia burgdorferi*, from black-legged tick, *Ixodes scapularis*, removed from a bird in Nova Scotia, Canada. Can Com Dis Rep. 1999;25:153–5.
24. Wilson ML, Telford SR III, Piesman J, Spielman A. Reduced abundance of immature *Ixodes dammini* (Acari: Ixodidae) following elimination of deer. J Med Entomol. 1988;25:224–8.
25. Rand PW, Lubelczyk C, Holman MS, Lacombe EH, Smith RP Jr. Abundance of *Ixodes scapularis* (Acari:Ixodidae) after the complete removal of deer from an isolated offshore island, endemic for Lyme disease. J Med Entomol. 2004;41:779–84.
26. Rand PW, Lubelczyk C, Lavigne GR, Elias S, Holman MS, Lacombe EH, et al. Deer density and the abundance of *Ixodes scapularis* (Acari:Ixodidae). J Med Entomol. 2003;40:179–84.
27. Kurtenbach K, Schaefer SM, Sewall H, Peacey M, Hoodless A, Nuttal PA, et al. Differential survival of Lyme borreliosis in ticks that feed on birds. Infect Immun. 2002;70:5893–5.
28. Dolan MC, Piesman J, Mbow ML, Maupin GO, Peter O, Brossard M, et al. Vector competence of *Ixodes scapularis* and *Ixodes ricinus* (Acari: Ixodidae) for three genospecies of *Borrelia burgdorferi*. J Med Entomol. 1998;35:465–70.

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Isolation of Lagos Bat Virus from Water Mongoose

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A genotype 2 lyssavirus, Lagos bat virus (LBV), was isolated from a terrestrial wildlife species (water mongoose) in August 2004 in the Durban area of the KwaZulu-Natal Province of South Africa. The virus isolate was confirmed as LBV by antigenic and genetic characterization, and the mongoose was identified as *Atilax paludinosus* by mitochondrial cytochrome *b* sequence analysis. Phylogenetic analysis demonstrated sequence homology with previous LBV isolates from South African bats. Studies performed in mice indicated that the peripheral pathogenicity of LBV had been underestimated in previous studies. Surveillance strategies for LBV in Africa must be improved to better understand the epidemiology of this virus and to make informed decisions on future vaccine strategies because evidence that current rabies vaccines provide protection against LBV is insufficient.

Lagos bat virus (LBV) belongs to the genus *Lyssavirus* in the family *Rhabdoviridae*. The prototype lyssavirus genotype and species, rabies virus (RABV), has a single, continuous, negative-strand RNA of ≈12,000 nt that codes for 5 proteins: nucleoprotein, matrix protein, phosphoprotein, glycoprotein, and polymerase (*1*). The *Lyssavirus* genus was created after isolation of several viruses in Africa and Europe that were related to, but serologically distinct from, RABV (*2*).

Seven genotypes (gts) or species in this genus are recognized (*3*), and diversity may expand with the addition of new isolates from Eurasia (*4*), which are tentative species in the *Lyssavirus* genus. RABV (gt1) is distributed worldwide, Australian bat lyssavirus (gt7) has only been identified in Australia, and European bat lyssavirus 1 (EBLV-1) (gt5) and European bat lyssavirus 2 (EBLV-2) (gt6) have been found only in Europe. Lagos bat virus (LBV) (gt2), Mokola virus (gt3), and Duvenhage virus (gt4) have been found only in Africa.

Recognized lyssavirus genotypes are divided into 2 serologically, pathogenically, and genetically distinct phylogroups (*5*). One phylogroup consists of Mokola virus and LBV (group II), while all other genotypes are in group I. Members of phylogroup I are reported to be pathogenic for mice when introduced intramuscularly and intracerebrally. In contrast, members of phylogroup II are believed to be pathogenic in mice only when introduced by the intracerebral (i.c.) route (*5*). Commercial vaccine strains belong to gt1 (RABV) phylogroup 1, and these vaccines provide protection against RABV and all the other members of phylogroup I. However, laboratory data suggest that these vaccines (gt1 based) will not offer protection against lyssaviruses in the phylogroup II cluster (*6*,*7*). On the basis of criteria proposed for lyssavirus phylogroups, West Caucasian bat virus could be considered an independent phylogroup III because of genetic distance and absence of serologic cross-reactivity with phylogroup I and II viruses (*7*).

LBV was first isolated from a fruit bat (*Eidolon helvum*) in 1956 on Lagos Island in Nigeria (*2*,*8*). Fourteen isolations of this virus have been reported throughout Africa, including 8 in South Africa (*9*). Most LBV isolates were obtained from bats; 2 were from domestic cats (*10*,*11*), and 1 was from a domestic dog in Ethiopia (*12*). LBV has never been isolated from any terrestrial wildlife species.

Globally and throughout Africa, RABV (gt1) is the most common lyssavirus. In southern Africa, 2 biotypes of RABV are recognized (*13*,*14*): the canid biotype, which mainly circulates among dogs, jackals, and bat-eared

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foxes, and the mongoose biotype, which is well adapted and unique to mongooses in southern Africa (*15*). RABV is responsible for all mongoose rabies cases in Africa. In South Africa, the principal vector of the mongoose biotype is the yellow mongoose (*Cynictis penicillata*), but RABV has been reported in other mongoose species, such as slender (*Galerella sanguinea*), water (*Atilax paludinosus*), small gray (*Galerella pulverulenta*), banded (*Mungos mungo*), selous (*Paracynictis selousi*), dwarf (*Helogale parvula*), and white-tailed (*Ichneumia albicauda*) mongooses. Mongoose rabies in South Africa commonly occurs in the central highveld regions (*15,16*), whereas KwaZulu-Natal Province, which is located on the east coast of South Africa, is associated with epizootic canid rabies in domestic dogs; mongoose rabies is not reported in this province.

We report the first identification of LBV in a wildlife terrestrial species, *A. paludinosus*, commonly known as the water or marsh mongoose. The mongoose species was identified by generation and analysis of cytochrome *b* sequencing data. We characterized this LBV isolate by antigenic typing with antinucleocapsid monoclonal antibodies, sequencing of the nucleoprotein gene, and peripheral pathogenicity in laboratory mice in comparison with other LBV isolates from South Africa and a bat RABV isolate from North America.

Materials and Methods

Sample Collection

In August 2004, a brain sample from a suspected rabid mongoose was submitted to the Allerton Veterinary Institute in Pietermaritzburg, KwaZulu-Natal, South Africa. The mongoose was captured by the Society for the Prevention of Cruelty to Animals in a marshy valley in a residential area in Westville near Durban after the mongoose displayed abnormal behavior. The animal was disorientated, attacked inanimate objects, and alternated between being friendly and aggressive. Only the brain of the animal was submitted for testing; the carcass was not preserved. The mongoose species was not identified.

Virus Characterization

Lyssavirus antigen was detected by the standard fluorescent antibody test (FAT) (*17*), with modifications, by using a polyclonal fluorescein isothiocyanate–conjugated immunoglobulin (Rabies Unit, Onderstepoort Veterinary Institute, Pretoria, South Africa) that could detect all lyssavirus genotypes. Virus isolation was performed by using the i.c. mouse inoculation test in suckling mice (*18*). Antigenic typing was performed by using the indirect fluorescent antibody test with a panel of 16 antinucleocapsid monoclonal antibodies (N-MAbs) (Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario, Canada) as previously described (*19*). Genetic characterization was based on sequencing of the entire nucleoprotein (N) gene.

Briefly, total RNA was extracted from infected brain material with Trizol (Invitrogen, Croningen, the Netherlands) according to the manufacturer's instructions. Complementary DNA was produced by a reverse transcription reaction by using an oligonucleotide primer specific for the noncoding messenger RNA of the lyssavirus genome (Lys001: 5′-ACGCTTAACGAMAAA-3′; position 1–15 according to the Pasteur virus [PV] RABV genome, GenBank accession no. M13215). Complementary DNA was amplified with a PCR by using different combinations of the oligonucleotide primers Lys001, LagNF (*9*), 550B (5′-GTRCTCCARTTAGCR CACAT-3′, position 647–666 according to the PV genome), and 304 (5′-TTGACAAAGATCTTGCTCAT-3′, position 1514–1533 according to the PV genome) as described elsewhere (*20*). The PCR products were visualized after electrophoresis on a 2% agarose gel and purified by using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). The purified products were sequenced with the BigDye Termination Cycle Sequencing Ready Reaction Kit 1.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol, with subsequent analysis on an Applied Biosystems 377 DNA automated sequencer.

Sequence Analysis

DNA sequencing information was compared with nucleoprotein sequence information for other lyssavirus genotypes in GenBank, as well as with nucleoprotein sequencing data obtained during this study from previous LBV isolates in South Africa from *Epomophorus whalbergi* fruit bats in 1980 (*21*), 1982 (*11*), 2003 (*9*), and 2004 (*9*), by using the same method as described above. CLUSTAL W (*22*) was used to produce sequence alignments and generate a neighbor-joining phylogenetic tree. A graphic representation of the tree was constructed with the TREEVIEW program (*23*).

Virus Pathogenicity

Two LBV isolates from South Africa (LBVSA2004) (*9*) and the LBV mongoose isolate described in this report (Mongoose2004), as well as a North American bat RABV (*Myotis* spp. variant, isolated in Washington, USA, 2004), were injected into 4-week-old inbred ICR mice (5 mice/group) by different routes. The i.c. 50% lethal dose (LD_{50}) was determined by titration of the virus suspension injected into 4-week-old ICR mice by the i.c. route. Thereafter, 4-week-old ICR mice were injected with 30 μL of 10^3 LD_{50} of each virus by the i.c. route and 30 μL of 10^5

LD_{50} of each virus by the intramuscular (i.m.) route. Virus inoculum was prepared by 1 i.c. passage of the original mongoose brain material in suckling mice.

Species Identification of the LBV-infected Mongoose

Because the mongoose carcass was destroyed, we attempted to accurately identify the animal by using DNA sequencing analyses of the mitochondrial cytochrome *b* region of mongoose genomic DNA obtained from the brain sample. The mitochondrial cytochrome *b* region has been used to characterize relationships between mongoose species (*24*). Genomic DNA was extracted from mongoose brain by using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), followed by PCR conducted according to the method of Veron et al. (*24*). PCR products were purified by using the Wizard SV PCR and gel purification kit (Promega) and sequenced by using the BigDye Termination Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems) according to the manufacturer's protocol, with subsequent analysis on an Applied Biosystems 3100 DNA automated sequencer. A DNA sequence of 893 bp of the cytochrome *b* gene was compared with cytochrome *b* sequences for mongooses available in GenBank by the same method as described earlier for the analysis of LBV nucleoprotein gene sequences.

Results

Virus Characterization and DNA Sequence Analysis

FAT performed on mongoose brain material showed a positive reaction for lyssavirus antigen. During the mouse inoculation test, suckling mice died 9 days after i.c. injections with mongoose brain suspensions. FAT of the suckling mouse brains showed a positive reaction for lyssavirus antigen. The isolate reacted with N-MAb 38HF2, which is an antibody that reacts with all lyssaviruses tested, and with the antibody N-MAb M612, which is highly specific for LBV and does not react with any other lyssaviruses tested. These findings indicate that the new isolate belongs to LBV (Table).

A reverse transcription–PCR method followed by a cycle sequencing method was used to amplify and determine the nucleic acid sequence of the entire nucleoprotein-encoding gene of the new putative LBV isolate. Phylogenetic analysis indicated that the new isolate clusters together with LBV isolates from South Africa and with LBV isolates from Nigeria (*2*) and Ethiopia (*12*) (Figure 1). LBV isolates from South Africa, including the new mongoose isolate of LBV, showed high nucleotide sequence identity with each other (99.1%–99.7%), compared with low sequence identity (≈82%) with the LBV isolate from Nigeria. The LBV isolate from Ethiopia (isolated from a dog; GenBank accession no. AY333110) showed 99.1%–99.9% nucleotide sequence homology with the South African LBV isolates. This result warrants further investigation of the DNA sequence identity of the Ethopian LBV isolate.

Virus Pathogenicity

Genotypes 1 and 2 viruses were pathogenic for mice by the i.c. and i.m. routes of injection (Figure 2). A similar death rate was observed for both genotypes (100%) after i.c. injection of equal amounts of virus (10^3 LD_{50} dose). Although the LBV isolates were lethal to mice when 10^5 LD_{50} was injected intramuscularly, they were less efficient than the RABV isolate. Of mice injected with the LBV

Table. Immunofluorescence patterns of 16 monoclonal antibodies with nucleoprotein of a new Lagos bat virus isolate from a mongoose in South Africa (Mongoose2004) compared with antigenic patterns of other South African lyssaviruses*

Antibody	RABV (canid biotype)	RABV (mongoose biotype)	LBV (bat isolates)	MOKV	DUVV	LBV (Mongoose 2004)
1C5	–	–	–	–	–	–
26AB7	+	Variable	–	–	–	–
26BE2	+	Variable	–	–	–	–
32GD12	Variable	Variable	–	–	–	–
38HF2	+	+	+	+	+	+
M612	–	–	+	–	–	+
M837	–	–	–	–	+	–
M850	–	Variable	–	–	+	–
M853	+	–	–	–	+	–
M1001	–	–	–	+	–	–
M1335	–	Variable	–	Variable	–	–
M1386	–	+	–	–	–	–
M1400	–	Variable	–	–	–	–
M1407	+	Variable	–	–	–	–
M1412	+	Variable	–	–	–	–
M1494	–	Variable	–	–	+	–

*Typical immunofluorescence antibody patterns observed for all lyssavirus genotypes present in Africa are included. RABV, rabies virus; LBV, Lagos bat virus; MOKV, Mokola virus; DUVV, Duvenhage virus; –, no fluorescence; +, fluorescence.

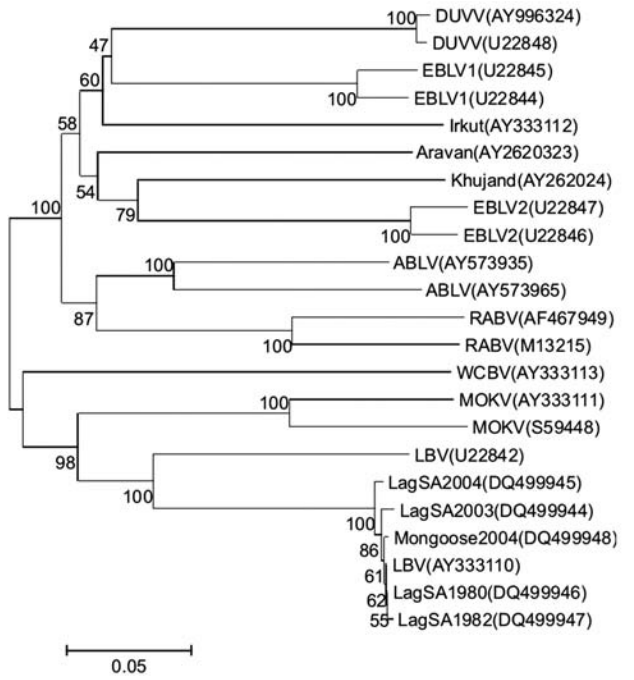

Figure 1. Neighbor-joining phylogenetic tree comparing nucleotide sequences of the entire nucleoprotein gene (1,350 nt) of a new Lagos bat virus (LBV) isolate from a mongoose in South Africa (Mongoose2004) and representative sequences of all other genotypes of lyssaviruses. Branch lengths are drawn to scale, and bootstrap values for 1,000 replicates are shown for the nodes. Accession numbers for all sequences available from GenBank and full-length nucleoprotein sequences of other LBV isolates from South Africa (1980, 1982, 2003, and 2004) are also included. DUVV, Duvenhage virus; EBLV, European bat lyssavirus; ABLV, Australian bat lyssavirus; RABV, rabies virus; WCBV, West Caucasian bat lyssavirus; MOKV, Mokola virus.

isolate from the mongoose, 20% died; 60% of the mice died after injection with the LBVSA2004 isolate from the fruit bat *E. whalbergi*. However, the RABV isolate showed 100% lethality in mice.

Species Identification of the LBV-infected Mongoose

Analysis of 893 bp of the cytochrome *b* gene obtained from mongoose brain indicated that the infected animal shared a 98% DNA nucleotide sequence homology with the African water mongoose (*A. paludinosus*) (Figure 3). Water mongooses are solitary and mainly nocturnal mammals, but they may also be active during the day. These animals live near water in areas with sufficient bush cover and have been found throughout sub-Saharan Africa (*25*).

Discussion

Isolation of LBV from terrestrial wildlife serves as further confirmation of our lack of understanding of the incidence and host range of lyssaviruses in Africa. Poor surveillance of rabies-related viruses and poor diagnostic capability in most of Africa are large contributors to our lack of information and the obscurity of the African lyssaviruses. The fluorescent antibody test used as a diagnostic test for rabies can only indicate the presence of lyssavirus antigens and cannot distinguish between lyssavirus genotypes. To identify a lyssavirus precisely, antigenic typing or genetic characterization is necessary, but these techniques are beyond the capability of most laboratories responsible for rabies diagnostics in Africa. Our phylogenetic analysis indicated a strong nucleoprotein sequence homology between LBV isolates from South Africa. Geographic partitioning is a well-known characteristic of RABV epidemiology worldwide. The strong sequence homology we observed may result from the defined geographic location from which all LBV isolates were obtained.

Although cases in domestic animals have been recorded, no human cases of infection with LBV have been documented. However, cross-neutralization data obtained with human sera and in rodent models suggest that preexposure and postexposure treatments for rabies are not effective against LBV (*6*,*7*). The infected mongoose showed aggressive behavior and was captured in a populated residential area. Although the incidence of the rabies-related viruses seems to be low, human exposure to these viruses is possible. Results of pathogenicity experiments indicated that death can occur from the i.c. and i.m. routes of injection, although gt2 viruses showed lower lethality to mice when injected i.m.

Our results differ from those of another study (*5*), which reported that a gt2 virus was not pathogenic to mice

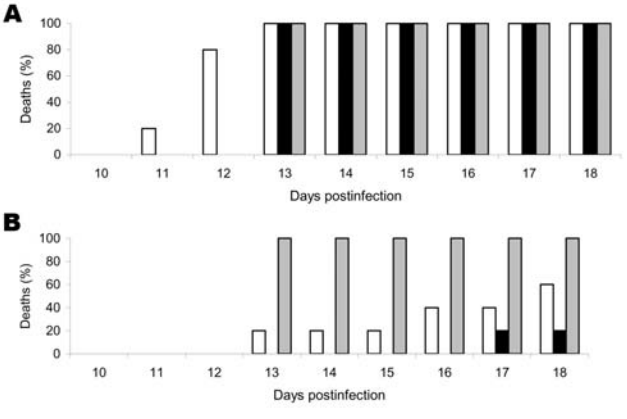

Figure 2. Pathogenicity of genotype 2 (LBVSA2004 [white bars] and Mongoose2004 [black bars]) and genotype 1 (gray bars) lyssaviruses in mice. Results are percentages of dead animals observed for a specific period. Mice were observed for 56 days, but no deaths occurred after 18 days. A) Deaths after intracerebral injection of 10^3 50% lethal doses (LD_{50}). B) Deaths after intramuscular injections of 10^5 LD_{50}.

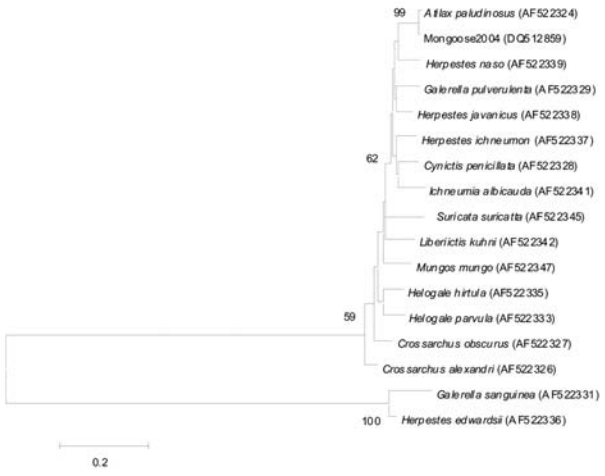

Figure 3. Neighbor-joining phylogenetic tree comparing 893 bp of the cytochrome *b* region of *Herpestidae* family sequences available in GenBank. Sequence obtained from the Lagos bat virus–infected mongoose (Mongoose2004) is 98% identical to the known cytochrome *b* sequences of *Atilax paludinosus* (water mongoose). GenBank accession numbers are indicated on the phylogenetic tree, and bootstrap values were determined with 1,000 replicates.

when administered by the i.m. route at the same dose (3×10^5 LD_{50}) used in our experiment. What amount of virus is involved in natural infection is not known. Cumulatively, our results indicate that LBV may be a health risk for humans and other mammals, and future vaccine strategies against rabies in Africa should consider these possibilities. Although laboratory data suggest little cross-neutralization of LBV by rabies preexposure and postexposure vaccination (*7*), immune system components other than neutralizing antibodies may be involved in protection. Therefore, in the absence of an alternative vaccine, rabies vaccination and postexposure treatment should still be advised because of potential cross-reactivity.

This report demonstrated the value of cytochrome *b* DNA sequencing for accurately identifying the host in a rabies case. Diagnostic laboratories do not routinely receive the complete carcass of suspected rabid animals, and identification is dependent on reports of persons who captured the animal or removed its brain before submission to the diagnostic facility. Host identity is rarely a problem in domestic animals, but wildlife species show potential uncertainty, such as demonstrated in the case reported here. One important aspect of disease epidemiology is accurate information about the host species involved, which enables informed decisions to be made with regard to the epidemiologic patterns and potential threats to public and veterinary health.

Identification of the first case of LBV in a mongoose underscores the need for surveillance of rabies-related viruses and the need for accurate identification of lyssavirus genotypes even if the host involved is normally only associated with RABV. With respect to LBV, we have recently reported the likely persistence of this virus in pteropid bats in South Africa, which implicates continuous opportunity for spillover into terrestrial species (*9*). In determining the extent of risk to human and veterinary public health, it is important to establish the prevalence of LBV not only in bats but also in potential terrestrial animal vectors, to which mongoose species should be added, based on the finding in this report.

The origin of mongoose rabies in South Africa is not clear (*14*). Epidemiologic cycles among yellow mongooses and other *Herpestidiae* are well established and shown to be impossible to extinguish or control by the attempted eradication or control of vector and host density (*26*). With respect to more modern or scientific approaches, no vaccination strategy has been considered feasible in tackling this complicated and entrenched wildlife rabies epidemic. Mongoose rabies may have originated from a spillover event of a bat lyssavirus progenitor in an event similar to the spillover described in this report.

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References

1. Tordo N, Poch O. Structure of rabies virus. In: Campbell JB, Charlton KM, editors. Rabies. Boston: Kluwer Academic Publishers; 1988. p. 25–45.
2. Boulger LR, Porterfield JS. Isolation of a virus from Nigerian fruit bats. Trans R Soc Trop Med Hyg. 1958;52:421–4.
3. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. Virus taxonomy: the classification and nomenclature of viruses. The eighth report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press; 2004. p. 623–31.
4. Kuzmin IV, Hughes GJ, Botvinkin AD, Orciari LA, Rupprecht CE. Phylogenetic relationships of Irkut and West Caucasian bat viruses within the *Lyssavirus* genus and suggested quantitative criteria based on the N gene sequence for lyssaviruses genotype definition. Virus Res. 2005;111:28–43.
5. Badrane H, Bahloul C, Perrin P, Tordo N. Evidence of two lyssavirus phylogroups with distinct pathogenicity and immunogenicity. J Virol. 2001;75:3268–76.
6. Nel LH. Vaccines for lyssaviruses other than rabies. Expert Rev Vaccines. 2005;4:533–40.

7. Hanlon CA, Kuzmin IV, Blanton JD, Weldon WC, Manangan JS, Rupprecht CE. Efficacy of rabies biologics against new lyssaviruses from Eurasia. Virus Res. 2005;111:44–54.
8. Shope RE, Murphy FA, Harrison AK, Causey OR, Kemp GE, Simpson DIH, et al. Two African viruses serologically and morphologically related to rabies virus. J Virol. 1970;6:690–2.
9. Markotter W, Randles J, Rupprecht CE, Sabeta CT, Taylor PJ, Wandeler AI, et al. 2006. Lagos bat, South Africa. Emerg Infect Dis. 2006;12:504–6.
10. King A, Crick J. Rabies-related viruses. In: Campbell JB, Charlton KM, editors. Rabies. Boston: Kluwer Academic Publishers; 1988. p. 177–200.
11. Crick J, Tignor GH, Moreno K. A new isolate of Lagos bat virus from the Republic of South Africa. Trans R Soc Trop Med Hyg. 1982;76:211–3.
12. Mebatsion T, Cox JH, Frost JW. Isolation and characterization of 115 street rabies virus isolates from Ethiopia by using monoclonal antibodies: identification of 2 isolates as Mokola and Lagos bat viruses. J Infect Dis. 1992;166:972–7.
13. Nel LH, Thomson GR, von Teichman BF. Molecular epidemiology of rabies virus in South Africa. Onderstepoort J Vet Res. 1993;60:301–6.
14. Von Teichman BF, Thomson GR, Meredith CD, Nel LH. Molecular epidemiology of rabies virus in South Africa: evidence for two distinct virus groups. J Gen Virol. 1995;76:73–82.
15. Nel LH, Sabeta CT, von Teichman B, Jaftha JB, Rupprecht CE, Bingham J. Mongoose rabies in southern Africa: a re-evaluation based on molecular epidemiology. Virus Res. 2005;109:165–73.
16. Swanepoel R. Rabies. In: Coetzer JA, Thomson GR, Tustin RC, Kriek NP, editors. Infectious diseases of livestock with special reference to southern Africa. Cape Town (South Africa): Oxford University Press; 1994. p.493–552.
17. Dean DJ, Abelseth MK, Atanasiu P. The fluorescence antibody test. In: Meslin FX, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies. 4th ed. Geneva: World Health Organization; 1996. p. 66–79.
18. Koprowski H. The mouse inoculation test. In: Meslin FX, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies. 4th ed. Geneva: World Health Organization; 1996. p. 88–95.
19. Wiktor TJ, Koprowski H. Monoclonal antibodies against rabies virus produced by somatic cell hybridization detection of antigenic variants. Proc Natl Acad Sci U S A. 1978;75:3938–42.
20. Sacramento D, Bourhy H, Tordo N. PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. Mol Cell Probes. 1991;5:229–40.
21. Meredith CD, Standing E. Lagos bat virus in South Africa. Lancet. 1981;1:832–3.
22. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80.
23. Page RD. Treeview: an application to display phylogenetic trees on personal computers. Comput Appl Biosci. 1996;12:357–8.
24. Veron G, Colyn M, Dunham AE, Taylor P, Gaubert P. Molecular systematics and origin of sociality in mongooses (Herpestidae, Carnivora). Mol Phylogenet Evol. 2004;30:582–98.
25. Estes RD. Behavior guide to African mammals. Berkeley (CA): University of California Press; 1991.
26. Snyman PS. The study and control of the vectors of rabies in South Africa. Onderstepoort J Vet Med Anim Husb. 1940;15:9–44.

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Modulatory Effect of Cattle on Risk for Lyme Disease

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To determine the effect of cattle on the risk for Lyme disease, we compared the prevalence of spirochete infection in questing vector ticks collected from a pasture with low-intensity cattle grazing with the prevalence in those collected from a site on which no cattle grazed. The presence of cattle limited the prevalence of *Borrelia burgdorferi* s.l., but not *B. miyamotoi*, in vector ticks. The reintroduction of traditional, nonintensive agriculture in central Europe may help reduce risk for Lyme disease.

The tick *Ixodes ricinus*, which transmits the spirochete agent of Lyme disease, is abundant in the ecotone at a forest's edge. The brushy vegetation there provides a suitable microclimate for these vector ticks as well as food and cover for the animals that serve as hosts for the ticks' subadult stages. Of those, rodents and particular passerine birds serve as reservoirs for the agent of Lyme disease (*1–8*). Other animals, such as domestic ungulates, serve as hosts for the tick but fail to support the pathogen. These animals exert a zooprophylactic effect on the transmission cycle by diverting ticks from feeding on reservoir-competent hosts. Any tick feeding on these hosts fails to acquire spirochetes and, thereby, fails to contribute to transmission (*9*,*10*). In addition, infected ticks may lose the pathogen when feeding on such incompetent hosts (*11–14*). The ecotone constitutes the focus of transmission, and persons may acquire numerous vector ticks when passing through such a site. The local composition of vertebrate animals serving as hosts to vector ticks determines the risk for infection at that site.

Recently, the European Union and the United Nations Educational, Scientific and Cultural Organization (UNESCO) instituted programs to limit the growth of brush and subsequent reforestation of uncultivated land. These programs specifically target the ecotonal habitat that developed ubiquitously in Europe when cultivating marginal land was considered too labor-intensive and costly. Toward this end, traditional farming methods are being reimplemented. Domestic ungulates, for example, are being introduced into the ecotone for landscape management. By grazing or browsing on scrub brush and saplings, these animals help maintain open landscapes and simultaneously enhance biodiversity. Although domestic animals may alter the contours of the ecotone, the effect of their presence on the risk for human infection by the agent of Lyme disease has not been defined.

To determine whether the presence of domestic ungulates may reduce risk for human Lyme disease, we compared the prevalence of spirochetal infection in ticks taken from an ecotonal cattle pasture to that in a site in which no cattle were permitted to graze. In addition, we determined the prevalence of the different Lyme disease genospecies in these ticks and that of a distantly related spirochete.

Materials and Methods

The study site, ≈2 km^2, is located near the town of Lembach, in the northern Vosges region of France, at the floodplain of the Sauer River. It is part of the Pfälzer Wald–Vosges du Nord biosphere reserve of UNESCO. Scottish Highland cattle were introduced there in 1999 for low-intensity grazing. About 250 cattle are allowed to graze in the floodplain year-round and are rotated on grazing patches enclosed by electric fencing on a total area of ≈200 km^2. The fence does not constitute a barrier to other vertebrates, such as hedgehogs, foxes, or deer.

On the cattle pasture and at a site 10 m outside the pasture, questing ticks were sampled by passing a flannel flag over the vegetation; ticks were preserved in 80% ethanol. We sampled the sites twice, in early May and early June 2005. Ticks were collected from 3 distinct areas within and 2 areas outside the pasture site. Both sites are separated by a path ≈4 m wide. Microscopy was used to identify stage and species of all ticks.

To detect and identify the various spirochetes that may be present in these ticks, the opisthosoma of each was opened, the contained mass of soft tissue was dissected out

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and placed in physiologic saline, and DNA was extracted as described previously (*15*). *Borrelia* genospecies were detected and characterized by amplifying and sequencing a 600-nt fragment of the gene encoding the 16S rRNA by nested PCR (*15*). Each resulting sequence was compared with sequences of the homologous gene fragment representing each of the validated spirochete genospecies. The following sequences served for comparison: GenBank accession nos. X85196 and X85203 for *B. burgdorferi* s.s.; X85190, X85192, and X85194 for *B. afzelii;* X85193, X85199, and M64311 for *B. garinii;* X98228 and X98229 for *B. lusitaniae;* X98232 and X98233 for *B. valaisiana;* AY147008 for *B. spielmanii;* and AY253149 for *B. miyamotoi*. A complete match, permitting no more than 2 nt changes, was required.

Results

To determine the effect of the presence of cattle on the prevalence of spirochetal infection in vector ticks, we sampled questing ticks within a fenced site where cattle grazed, examined them individually for spirochetal DNA by PCR and compared the resulting prevalence to that in ticks sampled from at least 10 m beyond the fence. Spirochetes infected ≈4× as many nymphs and ≈6× as many adults outside the enclosure as within (Table) (Fisher exact test, p<0.0001 for either developmental stage). The variance between different samples taken within the pasture site and the variance between samples taken outside the pasture were considerably smaller than the variance between the pasture and the nonpasture site. Fewer questing ticks harbored spirochetes where cattle were present than where they were absent.

We then determined whether the presence of cattle affects a spirochetal genospecies more than others. Amplified spirochetal DNA derived from each infected tick was sequenced and the genospecies determined by comparison with published sequences. Where cattle were present, virtually no nymphal or adult ticks were infected by *B. valaisiana* (Fisher exact test, p = 0.006, p = 0.05, respectively; Table). The prevalence of *B. lusitaniae* in ticks sampled on the cattle pasture was about one tenth the prevalence in ticks sampled outside the pasture (Fisher exact test, p = 0.025 in nymphs, p = 0.0025 in adults). *B. garinii* infected about one twentieth as many nymphal ticks and about one eighth as many adult ticks where cattle were present compared with the nonpasture site (Fisher exact test, p<0.0001 for either developmental stage). Whereas the prevalence of *B. afzelii* in nymphal ticks appeared to be not significantly affected by the presence of cattle, its prevalence in adult ticks was reduced to approximately one quarter (Fisher exact test, p = 0.0019). Too few ticks infected by *B. burgdorferi* s.s. or *B. spielmanii* were sampled to permit comparison. In contrast to the prevalence of Lyme disease spirochetes, the prevalence of *B. miyamotoi*, an ixodid-borne member of relapsing fever spirochetes, was similar, regardless of the presence of cattle. We conclude that the presence of cattle appears to reduce the prevalence of Lyme disease spirochetes in vector ticks but appears not to influence that of a distantly related spirochete.

Discussion

A vertebrate animal originally was said to be zooprophylactic if its presence in an endemic site serves to divert invertebrate vectors of a human pathogen from feeding on people (*16*). The concept was extended for Lyme disease to include more specific diversionary effects on the force of transmission of the pathogen, including diversion of the vector tick from feeding on potential reservoir hosts, transportation of vector ticks away from the venue of transmission, or elimination of infection from a previously infected tick (*11*). Lizards, for example, fail to support Lyme disease spirochetes, and the prevalence of infection in questing vector ticks appears reduced where such hosts are abundant (*2*,*9*). Ticks may detach from birds in nonsuitable habitats. Zooprophylaxis may affect the prevalence of infection in vector ticks by various factors.

Wild ungulates are generally thought to contribute to risk for Lyme disease, mainly by supporting the population of vector ticks. Indeed, adult *I. dammini* (or *scapularis*) ticks in the northeastern United States appear to preferably feed on white-tailed deer (*17*). Because they serve as definitive host for the tick, density of deer correlates with tick abundance (*18*,*19*). Reduction or elimination of deer

Table. Prevalence of spirochetes in questing nymphal and adult *Ixodes ricinus* ticks sampled in and outside a cattle pasture, France*

Cattle in site	Ticks examined				% Ticks infected by *Borrelia*						
	Stage	No.	% Infected	±SD	*B. afzeli*	*B. garinii*	*B. valaisiana*	*B. burgdorferi* s.s.	*B. lusitaniae*	*B. spielmanii*	*B. miyamotoi*
Present	Nymph	238	5.5	5.8	2.9	0.4	0	0.4	0.4	0	1.3
	Adult	136	5.9	3.7	3.7†	1.5	0	0	0.7	0	0.7
Absent	Nymph	95	22.1	5.8	5.3	9.5	4.2†	0	4.2	0	0
	Adult	236	40.3	2.3	13.6†	12.7	3.0‡	0.4	7.6	1.3	3.0

*SD, standard deviation.
†Includes a tick coinfected with *B. garinii.*
‡Includes 2 ticks coinfected with *B. garinii.*

on islands or their exclusion by electric fences has resulted in fewer subadult ticks questing in subsequent years; the density of adult ticks, however, has declined only slowly (*20*–*23*). In contrast, a simulation model indicates that the density of ticks may be more sensitive to the availability of hosts for the subadult stages than to that of hosts for adult ticks (*24*). In Europe, diverse medium-sized and large mammals, such as hares, hedgehogs, and foxes, constitute alternative hosts for adult ticks (*25*–*27*). As a result, any association between the abundance of vector ticks and the density of deer may be less profound in Europe than in eastern North America.

Domestic as well as wild ungulates exert a powerful zooprophylactic effect on Lyme disease spirochetes because they are incompetent for these pathogens. Not only do noninfected ticks that feed on these mammals fail to acquire spirochetes, but also infected ticks lose their infection during the course of such a bloodmeal (*10*,*12*,*28*,*29*). The sensitivity to the bacteriolytic activity of the complement pathway may determine the incompetence of a host (*30*). Cattle support the feeding of numerous ticks of all stages (*10*) and thereby divert subadult vector ticks from feeding on reservoir-competent hosts. A simulation model indicated that the availability of incompetent hosts for subadult tick stages would reduce prevalence of infection (*24*). Our observation that questing nymphal and adult ticks collected on a cattle pasture were less likely to be infected by Lyme disease spirochetes than were vector ticks collected outside the pasture supports this model. In addition, cattle and other domestic ungulates may modify the ecotonal vegetation by their grazing. This would render the microclimate more arid and, thereby, less suitable for the survival of ticks. Indeed, during equal time periods, we collected approximately half as many questing ticks where cattle were present as we did where cattle were absent (1 tick every 4 minutes vs. 2 minutes, respectively). Grazing cattle may also deplete reservoir-competent rodents and birds foraging in this ecotone of their food source and of cover from predators. By reducing the availability of potential reservoir hosts, additional subadult stages are diverted to feed on incompetent domestic ungulates. Whether the effect of cattle on the prevalence of spirochete infection in questing vector ticks results from the inability of cattle to support the pathogen or from their effect on tick habitat and reservoir rodents remains to be determined. In addition to their parasitologic effects, certain zooprophylactic animals may modify the ecotonal landscape in ways that affect transmission of the agent of Lyme disease.

Although the presence of cattle appears to suppress the prevalence of the various genospecies of Lyme disease spirochetes, that of *B. miyamotoi* is not affected. It is more closely related to spirochetes that cause relapsing fever than to spirochetes that cause Lyme disease (*31*). No disease relationship has yet been demonstrated for this microbe. *B. miyamotoi* spirochetes infect ≈2%–5% of questing nymphal or adult *I. ricinus*–like ticks, regardless of location (*15*,*32*). The prevalence of Lyme disease spirochetes, on the other hand, varies from zero to ≈50%, depending on the local composition of reservoir populations. Our observation that the prevalence of *B. miyamotoi* is insensitive to the presence of cattle corroborates reports on transovarial transmission (*32*) and suggests that this spirochete may cycle solely within the vector population.

The European Union and UNESCO support various policies for the deintensification of agriculture. The European Union's Biodiversity Action Plan for Agriculture, for example, recognizes that traditional nonintensive agriculture, as it was practiced for centuries until only a few decades ago, maintained an enormous species variety in wild and domestic flora and fauna (*33*). By encouraging extensive agricultural practices, such programs aim to prevent further abandonment of marginal farmland and the subsequent development of scrub brush and forests that support relatively few species. Management of previous fallow land as low-intensity pastures for robust varieties of cattle, sheep, goats, horses, or pigs helps maintain or reestablish not only open landscapes but also high nature value habitats (high biodiversity achieved by extensive farming). Our finding that the prevalence of Lyme disease spirochetes in questing vector ticks is significantly reduced on such pastures suggests that enhanced biodiversity may also contribute directly to human health. Conclusions derived from a mathematical model similarly suggest that a species-rich composition of vertebrates that serve as hosts for vector ticks would decrease the prevalence of spirochete-infected ticks by diluting the effect of reservoir-competent hosts (*34*). Efforts aimed at extensive landscape management may help to reduce risk for Lyme disease in central Europe.

We conclude that cattle appear to modulate risk for Lyme disease by reducing the prevalence of Lyme disease spirochetes in vector ticks; they do not, however, influence prevalence of a distantly related spirochete. Our observations were conducted in a high nature-value habitat that included an extensively managed cattle pasture. In addition to the direct parasitologic effects, certain zooprophylactic animals may modify the landscape in ways that affect the transmission of Lyme disease spirochetes. Removal of ground-covering vegetation may reduce the force of transmission of this pathogen by reducing local rodent density, thereby diverting vector ticks to reservoir-incompetent cattle. Zooprophylaxis may include environmental modification. Our finding that the prevalence of *B. miyamotoi* is insensitive to the presence of cattle suggests that this spirochete may be perpetuated differently

than are Lyme disease spirochetes. Extensive landscape management in central Europe may help reduce risk for Lyme disease.

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References

1. Donahue JG, Piesman J, Spielman A. Reservoir competence of white-footed mice for Lyme disease spirochetes. Am J Trop Med Hyg. 1987;36:92–6.
2. Matuschka F-R, Fischer P, Heiler M, Richter D, Spielman A. Capacity of European animals as reservoir hosts for the Lyme disease spirochete. J Infect Dis. 1992;165:479–83.
3. Matuschka F-R, Endepols S, Richter D, Spielman A. Competence of urban rats as reservoir hosts for Lyme disease spirochetes. J Med Entomol. 1997;34:489–93.
4. Matuschka F-R, Allgöwer R, Spielman A, Richter D. Characteristics of garden dormice that contribute to their capacity as reservoirs for Lyme disease spirochetes. Appl Environ Microbiol. 1999;65:707–11.
5. Olsen B, Jaenson TG, Bergstrom S. Prevalence of *Borrelia burgdorferi* sensu lato-infected ticks on migrating birds. Appl Environ Microbiol. 1995;61:3082–7.
6. Richter D, Spielman A, Komar N, Matuschka F-R. Competence of American robins as reservoir hosts for Lyme disease spirochetes. Emerg Infect Dis. 2000;6:133–8.
7. Tälleklint L, Jaenson TG. Transmission of *Borrelia burgdorferi* s.l. from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden. J Med Entomol. 1994;31:880–6.
8. Ginsberg HS, Buckley PA, Balmforth MG, Zhioua E, Mitra S, Buckley FG. Reservoir competence of native North American birds for the Lyme disease spirochete, *Borrelia burgdorferi*. J Med Entomol. 2005;42:445–9.
9. Lane RS, Loye JE. Lyme disease in California: Interrelationship of *Ixodes pacificus* (Acari: Ixodidae), the western fence lizard (*Sceloporus occidentalis*), and *Borrelia burgdorferi*. J Med Entomol. 1989;26:272–8.
10. Matuschka F-R, Eiffert H, Ohlenbusch A, Richter D, Schein E, Spielman A. Transmission of the agent of Lyme disease on a subtropical island. Trop Med Parasitol. 1994;45:39–44.
11. Matuschka F-R, Spielman A. Loss of Lyme disease spirochetes from *Ixodes ricinus* ticks feeding on European blackbirds. Exp Parasitol. 1992;74:151–8.
12. Matuschka F-R, Heiler M, Eiffert H, Fischer P, Lotter H, Spielman A. Diversionary role of hoofed game in the transmission of Lyme disease spirochetes. Am J Trop Med Hyg. 1993;48:693–9.
13. Telford SR, Mather TN, Moore SI, Wilson ML, Spielman A. Incompetence of deer as reservoirs of the Lyme disease spirochete. Am J Trop Med Hyg. 1988;39:105–9.
14. Jaenson TG, Tälleklint L. Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete. J Med Entomol. 1992;29:813–7.
15. Richter D, Schlee DB, Matuschka F-R. Relapsing fever-like spirochetes infecting European vector tick of Lyme disease agent. Emerg Infect Dis. 2003;9:697–701.
16. Russell PF, West LS, Manwell RD, MacDonald G. Practical malariology, 2nd ed. London: Oxford University Press; 1963.
17. Piesman J, Spielman A, Etkind P, Ruebush TK, Juranek DD. Role of deer in the epizootiology of *Babesia microti* in Massachusetts, USA. J Med Entomol. 1979;15:537–40.
18. Rand PW, Lubelczyk C, Lavigne GR, Elias S, Holman MS, Lacombe EH, et al. Deer density and the abundance of *Ixodes scapularis* (Acari: Ixodidae). J Med Entomol. 2003;40:179–84.
19. Wilson ML, Adler GH, Spielman A. Correlation between abundance of deer and that of the deer tick, *Ixodes dammini* (Acari: Ixodidae). Ann Entomol Soc Am. 1985;78:172–6.
20. Daniels TJ, Fish D. Effect of deer exclusion on the abundance of immature *Ixodes scapularis* (Acari: Ixodidae) parasitizing small and medium-sized mammals. J Med Entomol. 1995;32:5–11.
21. Rand PW, Lubelczyk C, Holman MS, Lacombe EH, Smith RP. Abundance of *Ixodes scapularis* (Acari: Ixodidae) after the complete removal of deer from an isolated offshore island, endemic for Lyme disease. J Med Entomol. 2004;41:779–84.
22. Stafford KC. Reduced abundance of *Ixodes scapularis* (Acari: Ixodidae) with exclusion of deer by electric fencing. J Med Entomol. 1993;30:986–96.
23. Wilson ML, Telford SR, Piesman J, Spielman A. Reduced abundance of immature *Ixodes dammini* (Acari: Ixodidae) following elimination of deer. J Med Entomol. 1988;25:224–8.
24. Van Buskirk J, Ostfeld RS. Controlling Lyme disease by modifying the density and species composition of tick hosts. Ecol Appl. 1995;5:1133–40.
25. Sréter T, Széll Z, Varga I. Ectoparasite infestations of red foxes (*Vulpes vulpes*) in Hungary. Vet Parasitol. 2003;115:349–54.
26. Tälleklint L, Jaenson TGT. Infestation of mammals by *Ixodes ricinus* ticks (Acari: Ixodidae) in south-central Sweden. Exp Appl Acarol. 1997;21:755–71.
27. Walter G, Liebisch A. Untersuchungen zur Biologie und Verbreitung von Zecken (Ixodoidea, Ixodidae) in Norddeutschland. III. *Ixodes ricinus* (Linnaeus 1758). Z Angew Zool. 1980;67:449–76.
28. Jaenson TGT, Tälleklint L. Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete. J Med Entomol. 1992;29:813–7.
29. Telford SR, Mather TN, Moore SI, Wilson ML, Spielman A. Incompetence of deer as reservoirs of the Lyme disease spirochete. Am J Trop Med Hyg. 1988;39:105–9.
30. Kurtenbach K, De Michelis S, Etti S, Schäfer SM, Sewell H-S, Brade V, et al. Host association of *Borrelia burgdorferi* sensu lato–the key role of host complement. Trends Microbiol. 2002;10:74–9.
31. Fukunaga M, Koreki Y. The flagellin gene of *Borrelia miyamotoi* sp. nov. and its phylogenetic relationship among *Borrelia* species. FEMS Microbiol Lett. 1995;134:255–8.
32. Scoles GA, Papero M, Beati L, Fish D. A relapsing fever group spirochete transmitted by *Ixodes scapularis* ticks. Vector Borne Zoonotic Dis. 2001;1:21–34.
33. Biodiversity action plan for agriculture. Communication from the commission to the council and the European parliament, vol. III; 2001 [cited 2006 Oct]. Commission of the European communities, Brussels, Belgium [online]. Available from http://ec.europa.eu/agriculture/envir/biodiv/162_en.pdf

34. LoGiudice K, Ostfeld RS, Schmidt KA, Keesing F. The ecology of infectious disease: Effects of host diversity and community composition on Lyme disease risk. Proc Natl Acad Sci U S A. 2003;100:567–71.

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Long-term Psychological and Occupational Effects of Providing Hospital Healthcare during SARS Outbreak

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Healthcare workers (HCWs) found the 2003 outbreak of severe acute respiratory syndrome (SARS) to be stressful, but the long-term impact is not known. From 13 to 26 months after the SARS outbreak, 769 HCWs at 9 Toronto hospitals that treated SARS patients and 4 Hamilton hospitals that did not treat SARS patients completed a survey of several adverse outcomes. Toronto HCWs reported significantly higher levels of burnout ($p = 0.019$), psychological distress ($p<0.001$), and posttraumatic stress ($p<0.001$). Toronto workers were more likely to have reduced patient contact and work hours and to report behavioral consequences of stress. Variance in adverse outcomes was explained by a protective effect of the perceived adequacy of training and support and by a provocative effect of maladaptive coping style and other individual factors. The results reinforce the value of effective staff support and training in preparation for future outbreaks.

Severe acute respiratory syndrome (SARS) emerged from Guangdong Province, People's Republic of China, in November 2002 and spread rapidly; transmission occurred primarily in hospitals, often to healthcare workers (HCWs). Although initially virtually no literature was available to guide expectations of how an emerging infection would affect the psychological well-being of hospital staff (*1*), by the summer of 2003 the acute psychological impact of SARS had been widely studied. Significant emotional distress was present in 18%–57% of HCWs (*2*–*6*) and was associated with quarantine (*7*), fear of contagion (*6*,*8*,*9*), concern for family (*5*,*9*,*10*), job stress (*6*,*9*), interpersonal isolation (*6*,*9*), perceived stigma (*6*,*7*,*11*), conscription of nonspecialists into infectious disease work (*12*), and attachment insecurity (*10*).

Working in SARS-affected hospitals could have been traumatic for some HCWs (i.e., an event that "threatens an individual's life or physical integrity and involves a subjective response of fear, helplessness, or horror" [*13*]). Before the SARS coronavirus was identified (*14*–*17*), SARS was an infection of unknown cause, unknown mode of transmission, global spread, and high mortality, characteristics that generally increase perceived risk (*18*). However, although the SARS outbreak was acutely stressful, the longer term impact of SARS on HCWs is unknown.

Understanding the enduring occupational and psychological effects of working during this SARS outbreak is important because it involves the well-being of large numbers of HCWs. Additionally, this information has wider relevance to health systems in planning for emerging infections, including pandemic influenza (http://www.who.int/csr/disease/influenza/inforesources/en) and the potential for bioterrorism (*19*). Although healthcare work

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during the SARS outbreak and during an influenza pandemic will differ in key respects, SARS experience provides the most extensive information available about the effects on HCWs of a large-scale infectious outbreak. The objective of the Impact of SARS Study was to assess the long-term psychological and occupational impact of SARS outbreak on HCWs and to identify personal and systemic factors that increase vulnerability.

Methods

Design, Setting, and Participants

The study took place in hospitals in Toronto and Hamilton in Ontario, Canada. Most of Canada's 438 suspected and probable SARS cases were identified in Toronto. Hamilton HCWs were selected as a comparison group because Hamilton is 57 km from Toronto and experienced all of the healthcare processes and precautions associated with Ontario's response to SARS (e.g., restrictions on access to care, protocols for staff screening, isolation procedures) but did not have SARS patients. Hamilton and Toronto hospitals are otherwise similar in terms of size, workload, and organizational characteristics. Thirteen participating sites (9 Toronto, 4 Hamilton) included academic and community hospitals. All Toronto sites treated SARS patients. Eligible HCWs included nurses in medical and surgical inpatient units and all staff of intensive care units, emergency departments, and SARS isolation units. Fifty-five clinical units participated (Toronto 40, Hamilton 15) from October 23, 2004, to September 30, 2005. This study was approved by the Research Ethics Board of each hospital.

Survey A measured adverse outcomes. All participants completed survey A anonymously and received Can $10. Those who were willing to provide more information participated in survey B, which measured potential mediators of adverse outcomes, and in 2 structured interviews (results to be reported elsewhere). Participants in survey B also received $50.

A separate "representativeness survey" was conducted from September through November 2005 to compare eligible Toronto HCWs who had participated in the Impact of SARS Study with those who had not. HCWs were approached at staff meetings in 14 participating clinical units and asked to complete an anonymous, 6-question questionnaire that surveyed whether or not they had participated in the Impact of SARS Study, exposure to SARS patients, age range, job type, years of healthcare experience, and overall subjective impact of SARS on their lives.

Measures

In the study instruments, "during the SARS outbreak" was defined for Toronto HCWs as the period from February 2003 to the day the last SARS patient was discharged from a participant's hospital or died. For Hamilton HCWs, the comparable period was defined as February through September 2003. SARS patients included probable and suspected SARS patients and persons isolated while their cases were under investigation for SARS according to the participants' report, rather than by using case definitions (http://www.phac-aspc.gc.ca/sars-sras/sarscasedef_e.html).

Survey A

This survey measured demographic and job data as well as traumatic stress response (15-item Impact of Events Scale [IES] [*20*,*21*]), nonspecific psychological distress (Kessler Psychological Distress Scale [K10] [*22*]), and professional burnout (emotional exhaustion scale of the Maslach Burnout Inventory [MBI-EE] [*23*–*25*]). To measure the practical and functional impact of SARS experience, participants were surveyed about changes since the SARS outbreak in healthcare work hours and the amount of face-to-face contact with patients in their work. Survey A also asked if survey participants had experienced an increase since the SARS outbreak in smoking, drinking alcohol, or "other activities that could interfere with your work or relationships" and how many work shifts had been missed in the 4 months preceding the survey because of stress, illness, or fatigue.

Survey B

Survey B, by using a previously described instrument, measured SARS-related perception of stigma and interpersonal avoidance; adequacy of training, protection, and support; and job stress (*6*,*10*,*26*). Scales calculated as the mean of all items related to these constructs showed adequate internal reliability (Table 1). Adaptive coping (problem-solving, seeking support, positive reappraisal) and maladaptive coping (escape-avoidance, self-blame, confrontative coping) regarding SARS were measured with the relevant subscales of the Ways of Coping Questionnaire (*27*), for which the stressful event was defined as the SARS outbreak. Attachment insecurity was measured with the anxiety and attachment avoidance scales of the Experiences in Close Relationships-Revised questionnaire (*28*).

Statistical Analysis

Central tendencies of parametric variables are described by mean and standard deviation; nonparametric variables are described by median and interquartile range. Between-group differences in parametric variables were determined by Student *t* test and in nonparametric variables by Mann-Whitney U test. To make the identified between-city differences more clinically meaningful, the

Table 1. Scales to measure perceptions about severe acute respiratory syndrome (SARS) experience

Scale	Perception
Training, protection and support* Cronbach α = 0.89	I had adequate training to deal confidently with the situations that I faced.
	Infection control procedures were adequately explained.
	I received adequate training in infection control procedures.
	I was provided with the protective equipment and procedures that I needed.
	I had someone to ask when I had problems using equipment.
	The hospital where I worked took my well-being into account when decisions were made that affected me.
	Emotional support (e.g., counseling) was available to those who needed help.
	I felt appreciated by the hospital/clinic/my employer.
	My hospital/workplace was supportive.
Job stress† Cronbach α = 0.76	There was more conflict among colleagues at work.
	I felt more stressed at work.
	I had to do work that normally I don't do.
	I had an increase workload.
	I had to work overtime.
Perceived stigma and interpersonal avoidance† Cronbach α = 0.77	I thought that people avoided me because of my profession.
	I thought that people avoided my family members because of my profession.
	I coped with the SARS situation by avoiding crowded places.
	I coped with the SARS situation by avoiding colleagues who might be exposed.

*Items scored on a 5-point scale from 1 (very confident that this is false) to 5 (very confident that this is true).
†Items scored on a 6-point scale from 1 (strongly disagree) to 6 (strongly agree).

prevalence of high scores was determined with standard cutoff values: IES $\geq$26 (http://www.mardihorowitz.com), MBI-EE $\geq$27 (*25*), K10 $\geq$16 (http://www.crufad.unsw.edu.au). Between-group differences in categorical variables were tested by χ^2.

To identify factors that might explain variance in adverse outcome, between-group differences in traumatic stress symptoms, psychological distress, and burnout were tested for the following categories: gender; duration of healthcare experience; job type; regular work during the SARS outbreak in emergency department, intensive care unit, or SARS isolation unit; indicators of the frequency and intensity of contact with SARS patients; and exposure to quarantine. A 10-day cutoff for quarantine was used, which corresponds to the standard period of quarantine for SARS (i.e., quarantine >10 days indicates extended quarantine or >1 period of quarantine). This analysis was performed in the full sample.

The relationship between adverse outcomes and potential mediating factors was identified by using Spearman rank-order correlations between adverse outcomes and measures of perceived systemic characteristics (stigma and interpersonal avoidance, adequacy of training, protection and support, and job stress) and psychological variables (coping style and attachment insecurity). This analysis was performed for survey A and B participants.

A stepwise regression analysis was performed for each adverse outcome. All potential mediating factors (those identified in the preceding univariate analyses with a significance of $p<0.05$) were entered. This analysis was performed for survey A and B participants.

Finally, to determine if factors that increase personal perceptions of risk had a practical functional impact on HCWs in the full sample, we identified an item in survey A that could serve as a proxy for the survey B factors that mediate vulnerability. This item is the duration (in months) of continuing perceived increased risk after the last SARS patient was discharged from a study participant's hospital or died. Duration of perceived risk was significantly correlated with the 2 SARS-specific mediating factors identified in the regression analysis: 1) maladaptive coping and perceived adequacy of training and 2) protection and support. For this analysis, the functional impact of SARS experience was operationalized as the number of adverse outcomes experienced by a person (from 0 to 7) of the following 7 outcomes: posttraumatic stress (IES $\geq$26); psychological distress (K10 $\geq$16); burnout (MBI-EE $\geq$27); decrease in face-to-face patient contact since SARS; decrease in work hours since SARS; increase in smoking, alcohol, or other problematic behavior since SARS; and $\geq$4 shifts missed because of stress or illness in the 4 months before the survey.

Results

In total, 1,984 HCWs received detailed information about the Impact of SARS Study and 769 (39%) completed survey A. The interval between the last SARS patient discharged or deceased and study participation was 13–25 (median 19) months.

To determine how representative participants were of all eligible hospital staff, after the Impact of SARS Study a representativeness study was presented to 258 Toronto HCWs who had been eligible; it was completed by 255 (99%) of these HCWs. Exposure to SARS patients was more common in HCWs who participated in the Impact of SARS Study than those who did not. However, study par-

ticipants and nonparticipants did not differ in age range, job type, years of healthcare experience, or overall subjective impact of SARS on their lives (Table 2).

Of the 769 participants, 73.5% were nurses (69.4% staff nurse, 3.1% manager or educator, 1.0% infection control practitioner). The next most prevalent job types were clerical staff (8.3%), physicians (2.9%), and respiratory therapists (2.3%). The remaining 99 participants (12.9%) were distributed among 14 different job types. Other characteristics of study participants, by city of employment, are presented in Table 3. Most Toronto participants (71.6%) reported contact with SARS patients, and Toronto participants were much more likely than Hamilton participants to have experienced quarantine (47.9% vs. 1.6%, p<0.001), which confirms the anticipated difference in SARS-related experience between comparison groups. A higher proportion of Hamilton participants were nurses (Hamilton 84.1% nurses vs. Toronto 71.2%, p = 0.001).

Survey B was completed by 187 HCWs (survey A and B participants). Survey A and B participants did not differ significantly from participants who only completed survey A by sex, job type (nurse or other), or city of employment. Survey A and B participants were older (mean 45 ± standard deviation 9 years vs. 41 ± 10 years, p<0.001) and more experienced in healthcare work (21 ± 10 years versus 16 ± 10 years, p<0.001). Survey A-only participants and Survey A and B participants did not differ with respect to exposure to SARS patients, working ≥5 shifts in intensive care unit, emergency department or SARS isolation unit during the outbreak or with respect to traumatic stress symptoms, psychological distress, or burnout.

During the study period (13–25 months after the SARS outbreak), Toronto HCWs reported significantly higher levels of burnout (Toronto median score 19, interquartile range 10–29; Hamilton 16, 9– 23, p = 0.019), psychological distress (Toronto 15, 12–19; Hamilton 13, 11–17, p<0.001), and posttraumatic stress (Toronto 11, 4–21; Hamilton 7, 0–19, p<0.001). To make these differences more clinically meaningful, the prevalence of high scores was calculated (Table 4). The prevalence of the following functional indicators of distress since the SARS outbreak was higher in Toronto HCWs: decrease in patient contact and work hours, increase in substance use and other traits that interfere with function, and more days off work (Table 4). Of the 7 adverse outcomes reported in Table 4, Toronto HCWs were more likely to be experiencing ≥1 problem (Toronto 68.1% vs. Hamilton 50.1%, p<0.001)) and were almost twice as likely to be experiencing multiple (≥2) problems (Toronto 44.0% vs. Hamilton 22.5%, p<0.001).

Personal and occupational characteristics of participants and the relationship of these variables to adverse outcomes are shown in Tables 5 and 6. Univariate relationships significant at the level of p<0.05 were retained for stepwise regression analysis to determine which of these variables accounted for significant variance in each adverse outcome (Table 7). Maladaptive coping and perceived adequacy of training together with protection and support explained 18% of the variance in burnout. The same 2 variables explained 21% of the variance in posttraumatic stress. Maladaptive coping and attachment anxiety, together with a protective effect of experience in healthcare, explained 31% of the variance in psychological distress.

Finally, the functional impact of vulnerability factors on the full survey A sample was tested by using duration of perceived risk after SARS as a proxy for the SARS-related vulnerability factors identified in the regression analysis. Duration of post-SARS perceived risk was correlated with maladaptive coping (Spearman ρ = 0.28, p = 0.001) and perceived adequacy of training, protection, and sup-

Table 2. Comparison of eligible Toronto healthcare workers who chose to participate or not to participate in the Impact of SARS Study*

Characteristics	Participation in Impact of SARS Study		p value
	Did not participate, % (n = 144)	Participated, % (n = 111)	
Age group, y			
<40	53	44	
≥40	47	56	0.17
Job type			
Nurse	73	71	
Other	27	29	0.76
Experience, y			
<10	51	41	
≥10	49	59	0.12
Treated SARS patient			
Yes	31	59	
No or don't know	69	41	<0.001
Overall impact			
Bad	40	50	
Neutral or good	60	50	0.11

*SARS, severe acute respiratory syndrome.

Table 3. Demographic and job characteristics of participants, Impact of SARS Study*

Characteristics	Toronto % (n = 587)	Hamilton % (n = 182)	p value
Female	86.0	89.6	0.22
Single	23.7	20.3	
Married or common-law	65.2	68.1	
Separated or widowed	11.1	11.5	0.41
Living with child ≤16 y of age	36.3	36.8	0.90
Living with adult ≥65 y of age	9.2	5.5	0.11
Worked in healthcare ≥10 y	65.1	68.7	0.37
Worked any shifts during SARS in			
Surgical inpatient unit	13.8	18.7	0.11
Medical inpatient unit	26.4	21.4	0.18
Isolation unit with SARS patients	22.5	†	
Intensive care unit	32.9	34.1	0.66
Emergency department	32.2	24.7	0.06

*SARS, severe acute respiratory syndrome.
†Hamilton had no patients with SARS.

port (Spearman $\rho = -0.27$, $p = 0.001$). The Figure shows a linear increase in the prevalence of multiple adverse outcomes in HCWs with longer duration of perceived risk. Duration of perceived risk and the overall number of adverse outcomes were significantly correlated. (Spearman $\rho = 0.23$, $p = 0.005$).

Discussion

This study highlights the resiliency of HCWs and, despite this trait, the potential that working during the SARS outbreak had a substantial negative impact on a statistically significant number of people. The evaluation of mediating factors suggests both systemic and individual targets for interventions to buffer the adverse effects of an extraordinary outbreak of infectious disease. Systemically, enhanced support and training may reduce burnout and posttraumatic stress. Individually, interventions that reduce maladaptive coping may decrease prolonged suffering.

The differences in adverse outcomes between Toronto and Hamilton HCWs were significant but small. However, further analysis suggests that the long-term impact of SARS has not been trivial. In particular, a categorical analysis (Table 4) shows that long-term adverse outcomes in Toronto HCWs occurred at a prevalence ≈50%–100% higher than in Hamilton HCWs. Furthermore, these outcomes may have a systemic impact, since SARS-affected HCWs reported reducing patient contact and hours of healthcare work as well as more frequent sick absences and an increase in behavior that could affect function.

These findings can be framed in terms of their potential value for the future. If the emergence of a new infectious disease is likely to increase the prevalence of significant distress in HCWs by 50%, to double the number of HCWs who are reducing their clinical practice or calling in sick, and if these difficulties will persist for ≥1–2 years after the outbreak's resolution, we want to learn from the SARS experience to try to buffer this negative impact. This discussion, therefore, addresses the identified mediators of SARS-related distress in HCWs and how these can guide preparation for pandemic influenza and other infectious disease outbreaks.

Exposure to high-intensity and high-risk work settings (such as intensive care units and emergency department work) and direct exposure to infected patients were not the primary determinants of adverse psychological outcomes. In fact, trends toward lower burnout in intensive care unit workers and less general psychological distress in emergency department workers were noted. These trends may be explained by the resilience of HCWs who choose this type of work and are consistent with the findings that longer healthcare experience was protective. We also found that the extent of various forms of distress was

Table 4. Prevalence of adverse outcomes in Hamilton and Toronto healthcare workers*

Adverse outcomes	Toronto, n = 587, %	Hamilton, n = 182, %	p value
High burnout (MBI-EE score ≥27)	30.4	19.2	0.003
High psychological distress (K10 score ≥16)	44.9	30.2	<0.001
High posttraumatic stress (IES score ≥26)	13.8	8.4	0.06
Since SARS have			
Decreased face-to-face patient contact	16.5	8.3	0.007
Decreased work hours	8.6	2.2	0.003
Increased smoking, drinking alcohol, or other behavior that could interfere with work or relationships	21.0	8.1	0.001
Missed ≥4 work shifts because of stress or illness	21.6%	12.6%	0.007

*MBI-EE, Maslach Burnout Inventory; K10, Kessler Psychological Distress Scale; IES, Impact of Events Scale; SARS, severe acute respiratory syndrome.

increased in Toronto HCWs, irrespective of their degree of contact with SARS patients, which implies that factors that are associated with the hospital environment as a whole and healthcare work in general during the outbreak were provocative.

Both systemic and personal variables were associated with persisting distress. In contrast to studies of distress during and shortly after the SARS outbreak (*6*,*9*,*12*), job stress related to conflict, workload, and conscription to new duties did not mediate long-term outcome. However,

Table 5. Relationship of healthcare worker, job, and SARS exposure characteristics to adverse outcomes in Toronto healthcare workers*

		Burnout			Psychological distress			Posttraumatic stress		
Characteristics	n	Median	Interquartile range	p value	Median	Interquartile range	p value	Median	Interquartile range	p value
Sex										
Male	82	18	9–29		14	12–19		10	2–19	
Female	505	19	10–29	0.30	15	12–19	0.91	12	4–21	0.02
Job type										
Nurse	418	21	11–29		14	11–18		12	5–22	
Other	169	14	8–27	0.002	15	12–20	0.16	10	2–19	0.1
Healthcare experience										
<10 y	205	21	12–30		16	12–21		11	11–21	
≥10 y	382	18	10–28	0.82	14	11–18	0.03	11	5–22	0.06
Worked on SARS unit										
<5 shifts	498	19	10–30		15	12–19		12	4–22	
≥5 shifts	89	17	11–26	0.75	15	11–20	0.54	10	3–17	0.63
Worked in ICU										
<5 shifts	427	20	10–30		15	12–19		11	4–21	
≥5 shifts	160	17	9–17	0.02	14	11–20	0.29	11	3–22	0.46
Worked in Emergency										
<5 shifts	434	18	10–28		15	12–20		12	5–21	
≥5 shifts	153	21	10–32	0.12	13	11–17	0.005	9	2–21	0.24
Ever in SARS patient room										
No	167	19	9–30		15	12–19		11	4–22	
Yes	420	19	10–28	0.33	15	11–19	0.09	12	4–21	0.16
Touched SARS patient										
No	265	19	9–30		15	11–19		12	4–22	
Yes	322	19	11–28	0.42	15	12–19	0.32	11	4–22	0.41
Protected contact with saliva or phlegm of SARS patient										
No	438	19	9–29		15	12–19		11	4–21	
Yes	149	19	11–29	0.43	15	12–18	0.78	10	4–22	0.44
Unprotected exposure to SARS patient										
No	502	18	9–28		15	11–19		11	4–21	
Yes	85	24	13–32	0.012	16	13–22	0.08	13	6–22	0.38
In SARS patients' rooms >5 min, >5 times										
No	316	18	9–28		15	11–18		11	3–21	
Yes	271	20	11–31	0.08	15	12–21	0.02	11	5–22	0.24
Quarantined										
Never	252	19	9–28		15	11–19		11	4–22	
≤10 d	235	17	10–28		15	11–19		11	3–21	
>10 d	100	21	11–34	0.36	16	12–22	0.09	13	5–22	0.42

*SARS, severe acute respiratory syndrome.

Table 6. Correlation between adverse outcomes after SARS and perceived characteristics of workplace and environment, coping style, and attachment insecurity in Toronto healthcare workers*

Characteristics of healthcare workers	Burnout		Psychological distress		Posttraumatic stress	
	Spearman ρ	p value	Spearman ρ	p value	Spearman ρ	p value
Training, protection and support	–0.297	<0.001	–0.162	0.06	–0.269	0.001
Stigma and avoidance	0.153	0.07	0.080	0.36	0.302	<0.001
Job stress	0.312	<0.001	0.224	0.008	0.164	0.052
Adaptive coping	0.066	0.44	0.147	0.08	0.182	0.03
Maladaptive coping	0.261	0.002	0.312	<0.001	0.364	<0.001
Attachment anxiety	0.179	0.049	0.355	<0.001	0.295	0.001
Attachment avoidance	0.078	0.40	0.204	0.03	0.139	0.13

*SARS, severe acute respiratory syndrome.

perceived adequacy of training, moral support, and protection were associated with better outcome. When the lessons of SARS are applied to pandemic planning, effective staff support may be a primary target to bolster the resilience of HCWs who will face future outbreaks. This observation is consistent with ones made during the SARS outbreak regarding the benefits of responsive communication (*29*), opportunities for facilitated reflection on normal emotional responses to extraordinary stress, and opportunities for HCWs to contribute to decision-making in the workplace (*10*,*30*).

Effective support benefits from careful planning and preparation before an outbreak, which the SARS situation did not allow. For example, effective moral or psychological support typically occurs in the context of trusted professional and institutional relationships, which should ideally be established before the outbreak situation. In particular, burnout has been identified as 1 of the most substantial health-related problems facing nurses (*31*). Because future outbreaks are likely to increase job strain and burnout, the prepandemic period is a critical time to attend to organizational characteristics that are known to buffer burnout, which include reducing patient-to-nurse ratios (*32*) and increasing organizational characteristics that increase nurses' autonomy, flexibility, control over practice (*33*), and perceived empowerment (*34*). The results of our study suggest that supportive interventions may be especially important for HCWs with fewer years of experience, who were more likely to experience prolonged psychological distress. Opportunities for mentorship or "buddying" with more experienced colleagues may be useful (*35*).

The personal variables that contributed to adverse outcomes were maladaptive coping through avoidance, hostile confrontation, and self-blame, and in the instance of general psychological distress, attachment anxiety. Although a review of interventions to modify coping style is beyond the scope of this paper, we note that organizational approaches to support staff and the individual experience of workers coping with extraordinary events are related. Hospital-based interventions to support staff may also promote adaptive coping. For example, engaging staff in collaborative planning for future outbreaks may reduce the tendency to cope by means of avoidant strategies and may enhance coping through problem-solving and peer-support. Anger and blame directed toward others (hostile confrontation) or oneself (self-blame) may be reduced in a working environment that fosters positive working rela-

Table 7. Variables that explain variance in adverse outcomes to severe acute respiratory syndrome (SARS) in Toronto healthcare workers

Variables	β	*t*	p value
Dependent variable: burnout*			
Maladaptive coping	0.29	3.34	0.001
Perceived adequacy of training, protection and support	–0.27	–3.10	0.002
Model R^2 = 0.18, p value <0.001			
Dependent variable: psychological distress†			
Maladaptive coping	0.31	3.78	<0.001
Years of healthcare experience	–0.26	–3.28	0.001
Attachment anxiety	0.24	2.87	0.005
Model R^2 = 0.31, p value <0.001			
Dependent variable: posttraumatic stress‡			
Maladaptive coping	0.37	4.39	<0.001
Perceived adequacy of training, protection and support	–0.22	–2.63	0.01
Model R^2 = 0.21, p value <0.001			

*Excluded variables: job stress, attachment anxiety, job type, worked in intensive care unit, unprotected contact with SARS patient(s).
†Excluded variables: job stress, attachment avoidance, worked in emergency department, in SARS patients room >5 min or >5 times.
‡Excluded variables: perceived stigma and avoidance, adaptive coping, attachment anxiety, job type, sex.

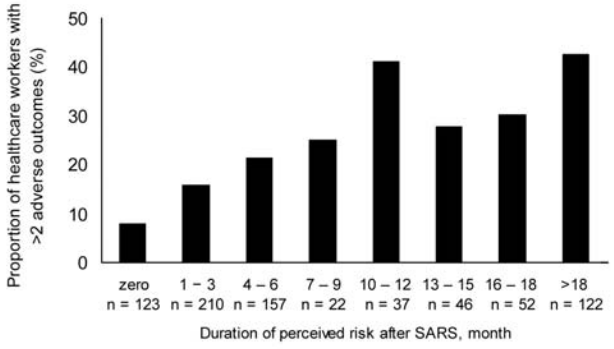

Figure. Relationship between prolonged perception of personal risk and reporting multiple adverse consequences of severe acute respiratory syndrome (SARS) in Toronto healthcare workers. Adverse outcomes are burnout; psychological distress; posttraumatic stress; decrease in face-to-face patient time since SARS; decrease in work hours since SARS; increase in smoking, drinking alcohol or other behavior that might interfere with work or relationships since SARS; and ≥4 work shifts missed because of stress or illness in the past 4 months.

tionships through effective leadership (*36*). Attachment anxiety is a common, relatively enduring, and stable interpersonal style within close relationships (*37*), which is known to be associated with sensitivity to stress under many conditions (*38*,*39*). Attachment anxiety is probably not a sensible target for hospital-based interventions to buffer the impact of systemic stresses, but it is a marker of those at greater risk for general psychological distress.

The results of this study also have implications for mitigating the effects of an infectious outbreak in the postoutbreak period. Because the duration of perceived risk in HCWs after the resolution of SARS is correlated with the severity of outcome, identifying and supporting HCWs who are at the highest risk for multiple and persistent psychological and occupational consequences of an outbreak may be possible by identifying HCWs whose perceived risk has not returned to normal within a few months after the event. Support programs, it would appear, need to be longer term to deal with ongoing residual effects after an outbreak. Programs directed toward healthy lifestyles, diet, exercise, and smoking cessation may also be important after the occurrence of an outbreak such as SARS to provide support to staff. Furthermore, for pandemic planning, the likelihood of prolonged subjective distress in a substantial percentage of HCWs should be factored into surge capacity modeling during and after the pandemic, particularly because distress is associated with reduced healthcare work.

Our conclusions are limited by the study method. With respect to generalizability, despite a response rate of 39%, the representativeness survey suggests that HCWs who participated were similar to nonparticipants. HCWs who had contact with SARS patients are overrepresented in the study sample, which may be because the study had greater salience for those persons, but study participants and nonparticipants did not differ in the subjective impact attributed to the SARS experience. A further limitation is that self-reports of SARS experiences do not provide an objective evaluation of actual differences in the training, protection, or support that HCWs received. Regardless of the limitations, the Impact of SARS Study provides a window on the long-term effects of working during times of extraordinary infectious risk.

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References

1. Maunder R, Hunter J, Vincent L, Bennett J, Peladeau N, Leszcz M, et al. The immediate psychological and occupational impact of the 2003 SARS outbreak in a teaching hospital. CMAJ. 2003;168:1245–51.
2. Tam CW, Pang EP, Lam LC, Chiu HF. Severe acute respiratory syndrome (SARS) in Hong Kong in 2003: stress and psychological impact among frontline healthcare workers. Psychol Med. 2004;34:1197–204.
3. Chan AO, Huak CY. Psychological impact of the 2003 severe acute respiratory syndrome outbreak on health care workers in a medium size regional general hospital in Singapore. Occup Med (Lond). 2004;54:190–6.
4. Phua DH, Tang HK, Tham KY. Coping responses of emergency physicians and nurses to the 2003 severe acute respiratory syndrome outbreak. Acad Emerg Med. 2005;12:322–8.
5. Nickell LA, Crighton EJ, Tracy CS, Al Enazy H, Bolaji Y, Hanjrah S, et al. Psychosocial effects of SARS on hospital staff: survey of a large tertiary care institution. CMAJ. 2004;170:793–8.
6. Maunder RG, Lancee WJ, Rourke S, Hunter JJ, Goldbloom D, Balderson K, et al. Factors associated with the psychological impact of severe acute respiratory syndrome on nurses and other hospital workers in Toronto. Psychosom Med. 2004;66:938–42.
7. Bai Y, Lin CC, Lin CY, Chen JY, Chue CM, Chou P. Survey of stress reactions among health care workers involved with the SARS outbreak. Psychiatr Serv. 2004;55:1055–7.
8. Ho SM, Kwong-Lo RS, Mak CW, Wong JS. Fear of severe acute respiratory syndrome (SARS) among health care workers. J Consult Clin Psychol. 2005;73:344–9.
9. Wong TW, Yau JK, Chan CL, Kwong RS, Ho SM, Lau CC, et al. The psychological impact of severe acute respiratory syndrome outbreak on healthcare workers in emergency departments and how they cope. Eur J Emerg Med. 2005;12:13–8.
10. Maunder R, Lancee WJ, Rourke SB, Hunter J, Goldbloom DS, Petryshen PM, et al. The experience of the 2003 SARS outbreak as a traumatic stress among frontline healthcare workers in Toronto: lessons learned. In: McLean AR, May RM, Pattison J, Weiss RA, editors. SARS: a case study in emerging infections. Oxford: Oxford University Press, 2005. p. 96–106.

11. Verma S, Mythily S, Chan YH, Deslypere JP, Teo EK, Chong SA. Post-SARS psychological morbidity and stigma among general practitioners and traditional Chinese medicine practitioners in Singapore. Ann Acad Med Singapore. 2004;33:743–8.
12. Chen CS, Wu HY, Yang P, Yen CF. Psychological distress of nurses in Taiwan who worked during the outbreak of SARS. Psychiatr Serv. 2005;56:76–9.
13. Yehuda R. Risk and resilience in posttraumatic stress disorder. J Clin Psychiatry. 2004;65(Suppl 1):29–36.
14. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003;348:1967–76.
15. Rota PA, Oberste MS, Monroe SS, Nix WA, Campagnoli R, Icenogle JP, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science. 2003;300:1394–9.
16. Kuiken T, Fouchier RA, Schutten M, Rimmelzwaan GF, van Amerongen G, van Riel D, et al. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. Lancet. 2003;362:263–70.
17. Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet. 2003;361:1319–25.
18. Slovic P. Perception of risk. Science. 1987;236:280–5.
19. Eisenman DP, Stein BD, Tanielian TL, Pincus HA. Terrorism's psychologic effects and their implications for primary care policy, research, and education. J Gen Intern Med. 2005;20:772–6.
20. Horowitz M, Wilner NJ, Alvarez W. Impact of Events Scale: a measure of subjective stress. Psychosom Med. 1979;41:209–18.
21. Horowitz MJ. Stress response syndromes: a review of posttraumatic and adjustment disorders. Hosp Community Psychiatry. 1986;37:241–9.
22. Kessler RC, Andrews G, Colpe LJ, Hiripi E, Mroczek DK, Normand SL, et al. Short screening scales to monitor population prevalences and trends in non-specific psychological distress. Psychol Med. 2002;32:959–76.
23. Maslach C, Jackson SE. Burnout in health professions: a psychological analysis. In: Snader GS, Suls J, editors. Social psychology of health and illness. Hillsdale (NJ): Erlbaum; 1982. p. 227–51.
24. Maslach C, Jackson SE. Maslach Burnout Inventory. Palo Alto (CA): Consulting Psychologists Press; 1986.
25. Maslach C, Jackson SE, Leitner MP. Maslach Burnout Inventory. 3rd ed. Third edition. In: Zalaquett CP, Woods RJ, editors. Evaluating stress: a book of resources. Lanham (MD): Scarecrow Press; 1997. p. 191–218.
26. Koh D, Lim MK, Chia SE, Ko SM, Qian F, Ng V, et al. Risk perception and impact of severe acute respiratory syndrome (SARS) on work and personal lives of healthcare workers in Singapore: What can we learn? Med Care. 2005;43:676–82.
27. Folkman S, Lazarus RS. Ways of coping questionnaire manual. Palo Alto (CA): Mind Garden; 1988.
28. Fraley RC, Waller NG, Brennan KA. An item response theory analysis of self-report measures of adult attachment. J Pers Soc Psychol. 2000;78:350–65.
29. Tseng HC, Chen TF, Chou SM. SARS: Key factors in crisis management. J Nurs Res. 2005;13:58–65.
30. Leszcz M. Group psychological perspectives on staff coping with SARS. New York: American Psychiatric Association Annual Meeting, May 6, 2004.
31. Kerr MS, Laschinger HKS, Severin CN, Almost JM, Shamian J. New strategies for monitoring the health of Canadian nurses: results of collaborations with key stakeholders. Can J Nurs Leadersh. 2005;18:67–81.
32. Aiken LH, Clarke SP, Sloane DM, Sochalski J, Silber JH. Hospital nurse staffing and patient mortality, nurse burnout, and job satisfaction. JAMA. 2002;288:1987–93.
33. Aiken LH, Clarke S, Sloane DM. Hospital staffing, organization, and quality of care: cross-national findings. Int J Qual Health Care. 2002;14:5–13.
34. Laschinger HKS, Finegan J, Shamian J, Wilk P. Impact of structural and psychological empowerment on job strain in nursing work settings: expanding Kanter's model. J Nurs Adm. 2001;31:260–72.
35. Guhde J. When orientation ends: supporting the new nurse who is struggling to succeed. J Nurses Staff Dev. 2005;21:145–9.
36. Yank GR, Barber JW, Hargrove DS, Whitt PD. The mental health treatment team as a work group: team dynamics and the role of the leader. Psychiatry. 1992;55:250–64.
37. Bowlby J. Attachment and loss: Vol 1: attachment. New York: Basic Books; 1969.
38. Mikulincer M, Florian V, Weller A. Attachment styles, coping strategies, and posttraumatic psychological distress: the impact of the Gulf War in Israel. J Pers Soc Psychol. 1993;64:817–26.
39. Maunder RG, Lancee WJ, Nolan RP, Hunter JJ, Tannenbaum DW. The relationship of attachment insecurity to subjective stress and autonomic function during standardized acute stress in healthy adults. J Psychosom Res. 2006;60:283–90.

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Methicillin-resistant *Staphylococcus aureus* Colonization in Veterinary Personnel

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Methicillin-resistant *Staphylococcus aureus* (MRSA) was isolated from nares of 27/417 (6.5%) attendees at an international veterinary conference: 23/345 (7.0%) veterinarians, 4/34 (12.0%) technicians, and 0/38 others. Colonization was more common for large-animal (15/96, 15.6%) than small-animal personnel (12/271, 4.4%) or those with no animal patient contact (0/50) (p<0.001). Large-animal practice was the only variable significantly associated with colonization (odds ratio 2.9; 95% confidence interval 1.2–6.6). Pulsed-field gel electrophoresis identified 2 predominant clones with similar distribution among veterinarians as previously reported for horses and companion animals. Canadian epidemic MRSA-2 (CMRSA) was isolated from 11 small-animal and 2 large-animal personnel from the United States (n = 12) and Germany (n = 1). In contrast, CMRSA-5 was isolated exclusively from large-animal personnel (p<0.001) in the United States (n = 10), United Kingdom (n = 2), and Denmark (n = 1). MRSA colonization may be an occupational risk for veterinary professionals.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a problematic pathogen in human medicine and appears to be an emerging problem in veterinary medicine. Historically, hospital-associated MRSA infections have predominated in humans and contributed to significant illness and death (*1–4*). Recently, a shift in the epidemiology of MRSA infection has been documented, whereby community-associated (CA)-MRSA infections have become more common (*5–9*). CA-MRSA may arise from hospital-origin clones that are carried into the community and then transmitted between persons or from de novo development of resistance through acquisition of resistance factors (*mec*A) by methicillin-sensitive strains of *S. aureus* (*10*). Asymptomatic colonization with MRSA represents a major risk factor for infection or for transmission among persons within hospitals or the community (*11*).

While CA-MRSA infections are becoming more widely reported, the prevalence of MRSA carriage overall remains low in healthy persons in the community (*6*,*12–14*). Reported prevalence of MRSA colonization in the community has been variable; the study population has a major effect on MRSA carriage rates. In the absence of recognized risk factors, the prevalence of colonization tends to be low. In a 2003 study, Salgado et al. identified MRSA colonization in 1.3% of persons overall but in only 0.2% of persons with no identified healthcare-associated risk factors (*12*). A study from Switzerland reported MRSA colonization in 0.09% of persons at the time of hospital admission (*6*). The prevalence of MRSA carriage was 0.3% in a 2005 study that Nulens et al. conducted at a European conference for physicians and others involved in clinical microbiology and infectious disease (*15*).

MRSA infection and colonization have been reported in horses, dogs, cats, birds, and cattle (*16–19*). Transmission of MRSA between animals and humans has been reported (*20–23*) as have human MRSA infections from animal contact (*16*,*21*,*24*). Recent studies have identified high colonization rates in humans who have close contact with animals. MRSA colonization of persons who work with horses in Canada and the United States was 13% (14/107); on every farm where MRSA was identified in a horse, at least 1 person was colonized (*25*). In another study, 10 (9.7%) of 103 tested veterinary hospital personnel in a large-animal clinic were colonized with MRSA, and clinical skin infections were reported in 3 (*26*). Isolates from horses and humans in each of these studies were indistinguishable by pulsed-field gel electrophoresis (PFGE) and

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were typed as Canadian epidemic MRSA (CMRSA)-5 (ST8:MRSA:*SCCmecIV*, also known as USA500), which suggests transmission between horses and humans (*27*). A study at a small-animal referral hospital in the United Kingdom reported MRSA colonization in 17.9% of veterinary personnel. Investigation of clinical infection in 5 dogs and 3 cats found colonization in 14 (16%) of 88 household contacts or veterinary personnel (*28*). In all of the above reports, a screening bias for MRSA colonization may have been present if an outbreak had been ongoing in the population. Whether these results would accurately reflect the prevalence of MRSA in the general veterinary population, and therefore the occupational risk of MRSA exposure for veterinarians, is unclear. Our objective was to determine the prevalence of MRSA colonization in veterinary personnel attending an international veterinary conference and to characterize recovered MRSA isolates.

Materials and Methods

Study Population

This study was performed at the annual American College of Veterinary Internal Medicine Forum held in Baltimore, Maryland, USA, June 3–5, 2005. The conference was attended by 3,240 persons: 2,744 practicing veterinarians, 354 technicians, and 142 other veterinary personnel involved in industry or research. Most (86%) attendees were from the United States; however, 43 other countries were represented. An information and sampling booth attended by the investigators was used to enroll adult volunteers; all attendees were eligible. This study was approved by the University of Guelph Research Ethics Board.

Sample Collection

Participants provided a single nasal swab sample each, which they collected themselves according to instructions to insert a cotton-tipped swab ≈1 cm into each nostril. The swabs were placed in liquid Stuart medium and maintained at 4°C until processing.

Participants completed a brief questionnaire designed to identify potential risk factors for MRSA colonization: nationality, occupational position, type of clinical practice, veterinary patient contact, known exposure to MRSA in veterinary practice, previous hospitalization (within 30 days), previous MRSA infection, and residence with a healthcare worker. Practice types were small-animal (primarily dogs and cats), large-animal (primarily horses but also ruminants), and mixed (combination of large and small animals). We defined CA-MRSA colonization as MRSA isolation from a person with no history of healthcare-associated risk factors.

MRSA Identification, Characterization, and Typing

Swabs were placed into 2 mL of enrichment broth consisting of 10 g/L Tryptone T (Oxoid Inc., Nepean, Ontario, Canada), 75 g/L sodium chloride, 10 g/L mannitol, and 2.5 g/L yeast extract and incubated for 24 h at 35°C. Approximately 100 μL of broth was spread onto mannitol-salt agar with 10 g/L cefoxitin and incubated at 35°C for 48 h. Isolates were identified as *S. aureus* on the basis of colony morphologic features, gram-positive stain, catalase-positive reaction, positive tube coagulase test result, and positive latex agglutination test result (Pastorex Staph Plus, Bio-Rad Laboratories Ltd, Mississauga, Ontario, Canada). Methicillin-resistance was confirmed by demonstration of penicillin binding protein 2a with a latex agglutination antibody screening kit (Denka Seinken Co. Ltd, Tokyo, Japan). Antimicrobial susceptibility was performed by Kirby-Bauer disk diffusion according to the Clinical Laboratory Standards Institute (CLSI) guidelines (*29*); mupirocin MIC was determined by using E-Test gradient strips (AB Biodisk, Solna, Sweden). MRSA isolates were typed by *Sma*I PFGE and categorized as different CMRSA types as described previously (*8*). Real-time PCR was used to detect the *lukF* and *lukS* components of the Panton-Valentine leukocidin (PVL) gene previously described (*30*).

Statistical Analysis

Categorical comparisons were performed using χ^2 analysis or Fisher exact test. A p value <0.05 was considered significant for all comparisons. Risk factors for MRSA colonization were evaluated by using stepwise forward logistic regression. Variables achieving a liberal significance level of $p \leq 0.20$ in the univariate analyses were considered for inclusion in the multivariate model. The presence of confounding was evaluated by noting the effect of elimination on the coefficients of the remaining variables. Variables achieving $p<0.05$ in the final model were considered significant, and odds ratios (ORs) with 95% confidence intervals (CIs) were calculated.

Results

Nasal swabs were provided by 417 attendees from 19 countries. MRSA was isolated from the nasal cavities of 27 (6.5%) of 417 persons: 15 (15.6%) of 96 in large-animal practice, 12 (4.4%) of 271 in small-animal practice, and 0 of 50 in industry or research ($p<0.001$) (Table 1). The prevalence of colonization was higher in men (13/139, 9.4%) than in women (14/265, 5.3%); however, this difference was not statistically significant ($p = 0.09$).

Colonized persons were from the United States (n = 23), United Kingdom (n = 2), Denmark (n = 1), and Germany (n = 1). Difference in the prevalence of colonization between countries was not significant ($p = 0.18$).

Table 1. Risk factors for MRSA colonization among veterinary conference attendees, Baltimore, Maryland, USA, June 3–5, 2005*

Variable	MRSA-colonized persons, n/N (%)	p value
In clinical practice		0.01
Yes	27/367 (7.4)	
No	0/47	
Animal type†		<0.01
Large	15/96 (15.6)	
Small	12/271 (4.4)	
Practice/facility type		
Academic	11/136 (8.1)	0.29
Private specialty	10/103 (10)	0.10
General	6/139 (4.3)	0.97
Other	0/36	0.97
Position		0.96
Veterinarian	23/345 (7.0)	
Technician	4/34 (12.0)	
Personally diagnosed MRSA in an animal		0.12
Yes	19/275 (6.9)	
No	7/48 (13)	
MRSA case identified at clinic		0.045
Yes	15/244 (6.2)	
No	12/97 (12)	
Personally had diagnosis of MRSA infection or colonization		0.98
Yes	0/4	
No	27/363 (7.4)	
Hospitalized within past 30 d		0.28
Yes	1/5 (20)	
No	26/362 (7.2)	
Healthcare worker in household		0.81
Yes	2/33 (6.1)	
No	25/309 (7.5)	

*MRSA, methicillin-resistant *Staphylococcus aureus*.
†Clinical practice only.

Colonization rates in persons from the United States, Canada, and the United Kingdom—the 3 countries with the highest representation—were 6.0%, 0%, and 17%, respectively (p = 0.11). According to stepwise forward logistic regression, employment in a large-animal practice was the only variable independently associated with MRSA colonization (OR 2.9; 95% CI 1.2–6.6).

Characterization of MRSA isolates, using PFGE, identified 2 predominant clones: CMRSA-5 (ST8-MRSA-IV, similar to USA500) and CMRSA-2 (ST5-MRSA-II, similar to USA100) (*8*,*31*). CMRSA-5 was isolated from 13 (48%) of 27 colonized persons, all of whom were in large-animal practice. These persons were from the United States (n = 10), United Kingdom (n = 2), and Denmark (n = 1). CMRSA-2 was isolated from 13 (48%) of 27 colonized persons: 11 in small-animal practice and 2 in large-animal practice from the United States (n = 12) and Germany (n = 1). One other isolate, possibly related to CMRSA-2, was recovered from a US veterinarian in small-animal practice. Overall, CMRSA-5 was more commonly isolated from persons in large- than in small-animal practice (p<0.001). A cluster of 5 colonized persons was identified from a US school of veterinary medicine, where 4 persons who worked with large animals were colonized with CMRSA-5, and 1 veterinarian who worked with small animals was colonized with CMRSA-2. No isolates were identified as carrying the *lukF* or *lukS* genes. All isolates were susceptible to vancomycin; other antimicrobial drug susceptibility results are shown in Table 2.

Discussion

This study represents the most comprehensive evaluation to date of MRSA colonization in veterinary personnel. Although a control group was not included, the prevalence in veterinary personnel (6.5%) was higher than previously reported rates for community-based colonization (*6*,*12–14*,*32*). When compared with results from a similar study in which only 0.3% of medical professionals at a conference were colonized (*15*), our results suggest an increased risk for veterinary professionals. However, geographic location and culture methods may have affected the differences in study results, and further investigation is required to more accurately define the occupational risk.

The PFGE type distribution provides support that MRSA is being transmitted between animals and humans. A significant difference was identified between clones

Table 2. Antimicrobial susceptibility of MRSA isolates recovered from veterinary conference attendees, Baltimore, Maryland, USA, June 3–5, 2005*

PFGE type	No.	Susceptible isolates, n (%)						
		Mupirocin	Erythromycin	Ciprofloxacin	Tetracycline	Gentamicin	Rifampin	TMP/SMX
CMRSA-2	13	13 (100)	1 (7.7)	2 (15)	13 (100)	12 (92)	13 (100)	13 (100)
CMRSA-5	13	13 (100)	6 (46)	12 (92)	2 (15)	3 (23)	8 (62)	3 (23)
Other	1	0	0	0	1 (100)	1 (100)	1 (100)	1 (100)
Total	27	26 (96)	7 (26)	14 (52)	16 (59)	16 (59)	22 (81)	17 (63)

*MRSA, methicillin-resistant *Staphylococcus aureus;* PFGE, pulsed-field gel electrophoresis; TMP/SMX, trimethoprim/sulfamethoxazole; CMRSA, Canadian epidemic MRSA.

recovered from those who worked with large animals (CMRSA-5) and those who worked with small animals (CMRSA-2). If these results merely represented the background level of CA-MRSA colonization in the general population, this difference would not be expected. In addition, CMRSA-5 has not been commonly identified in persons in the community, at least not in Canada (*8*,*33*), although it has accounted for most of the reported cases of MRSA infection or colonization in horses (*16*,*24*,*27*). These findings, along with the identification of CMRSA-5 in large-animal veterinarians from the United States, United Kingdom, and Denmark, provide further evidence that CMRSA-5 is widely disseminated in the horse population and may be transmitted between horses and humans. The CMRSA-5 isolate from the Danish veterinarian was further classified as sequence type 8 and Ridom spa type t064, which has been identified in humans in Denmark, Norway, Germany, Belgium, and Sweden (R. Skov, pers. comm.). CMRSA-5 predominance in large-animal veterinarians may infer an occupational health concern for veterinary professionals who have contact with equine patients. Furthermore, this study's finding of large-animal practice as the only variable associated with colonization and the striking prevalence of MRSA colonization in persons who work with large animals (15.6%) also support an occupational risk for MRSA exposure in large-animal practice. Because zoonotic infections have been associated with exposure to CMRSA-5 (*26*), further evaluation of interspecies transmission within large-animal veterinary practices by routine screening of patients and personnel, along with implementation of infection control practices, may be warranted.

Identification of CMRSA-2 primarily in small-animal veterinarians was consistent with previous reports that MRSA isolates from dogs and cats reflect the predominant human strains in the community (*17*,*22*). In Canada, CMRSA-2 is a hospital-origin clone that has emerged in the community as a major cause of CA-MRSA infections in people (*33*,*34*). The lower prevalence of colonization in persons who work with small animals and the predominance of a common community clone make it more difficult to implicate animal contact as the source of MRSA. However, because colonization in persons who work with small animals was higher than community prevalence rates and because MRSA transmission from household pets to humans has been reported (*20*,*21*), dogs and cats must be considered as possible sources of MRSA. A comprehensive evaluation of MRSA colonization in small animals and risk factors for interspecies transmission are required to determine the true occupational risk for MRSA colonization and infection in small-animal veterinary personnel (*17*,*18*).

Although CA-MRSA isolates that express the PVL genes are frequently a cause of severe skin and soft tissue infections in the community (*8*,*9*,*35–37*), this virulence factor was not identified in isolates from veterinary personnel. These results are similar to those of previous studies in which MRSA isolates from veterinary species or personnel have been negative for PVL genes (*24*,*26*,*28*,*38*). However, considering the recent dissemination of the PVL-positive USA300 clone throughout North America and that MRSA isolates in dogs and cats often reflect the predominant community clones, PVL-positive clones may emerge in small animals (*36*). Further monitoring of the dynamic epidemiology of CA-MRSA is required to determine whether animals will have any role in dissemination of this clone.

Previous contact with MRSA in a colonized or infected animal was reported by 6.9% of colonized persons but was not significantly associated with MRSA colonization in veterinary personnel. The number of personnel reporting previous contact with an infected animal was low, thereby limiting the ability of this study to identify an association. Intuitively, one would assume that contact with MRSA-infected animals in veterinary practice would represent a high-risk situation; further investigation is required to more accurately determine this risk.

This study has several limitations. First, the sample used was a convenience sample of attendees at the Forum, which allows potential sample bias. Second, a greater proportion of veterinary personnel sampled were in specialty practice, leading to results that, because of different patient populations, may not apply to general practitioners. Third, because this was a cross-sectional study, causality between risk factors and colonization could not be determined; only an association between variables and colonization could be implied. The small sample size from several countries also limited the statistical power to identify an association in these populations. Lastly, because attendees collected their

own nasal swabs, sampling variation may have led to underestimation of the prevalence of colonization. The variable sensitivity (75%–93%) of using nasal swab screening alone (*39*) could also have led to false-negative results. Ideally, a randomized sample of general and specialty practitioners would be performed using >1 sampling site to further characterize the prevalence of MRSA colonization in veterinary personnel.

As MRSA expands into the community, changes in its epidemiology are inevitable. The lives of humans and animals, and their microflora, are closely intertwined. MRSA is now a pathogen of domestic animals that can be transmitted between animals and humans. Accordingly, further scrutiny of the roles of animals in MRSA infection and colonization is required. While occupational and recreational exposure to horses may be a risk factor for MRSA colonization, the effect of routine contact with household pets on the global epidemiology of MRSA is still unknown.

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References

1. Kuehnert MJ, Hill HA, Kupronis BA, Tokars JI, Solomon SL, Jernigan DB. Methicillin-resistant *Staphylococcus aureus* hospitalizations, United States. Emerg Infect Dis. 2005;11:868–72.
2. Osawa K, Baba C, Ishimoto T, Chida T, Okamura N, Miyake S, et al. Significance of methicillin-resistant *Staphylococcus aureus* (MRSA) survey in a university teaching hospital. J Infect Chemother. 2003;9:172–7.
3. Marshall C, Kossmann T, Wesselingh S, Spelman D. Methicillin-resistant *Staphylococcus aureus* and beyond: what's new in the world of the golden staph? ANZ J Surg. 2004;74:465–9.
4. Tiemersma EW, Bronzwaer SL, Lyytikainen O, Degener JE, Schrijnemakers P, Bruinsma N, et al. Methicillin-resistant *Staphylococcus aureus* in Europe, 1999–2002. Emerg Infect Dis. 2004;10:1627–34.
5. Faria NA, Oliveira DC, Westh H, Monnet DL, Larsen AR, Skov R, et al. Epidemiology of emerging methicillin-resistant *Staphylococcus aureus* (MRSA) in Denmark: a nationwide study in a country with low prevalence of MRSA infection. J Clin Microbiol. 2005;43:1836–42.
6. Harbarth S, Francois P, Shrenzel J, Fankhauser-Rodriguez C, Hugonnet S, Koessler T, et al. Community-associated methicillin-resistant *Staphylococcus aureus*, Switzerland. Emerg Infect Dis. 2005;11:962–5.
7. Ochoa TJ, Mohr J, Wanger A, Murphy JR, Heresi GP. Community-associated methicillin-resistant *Staphylococcus aureus* in pediatric patients. Emerg Infect Dis. 2005;11:966–8.
8. Mulvey MR, MacDougall L, Cholin B, Horsman G, Fidyk M, Woods S. Community-associated methicillin-resistant *Staphylococcus aureus*, Canada. Emerg Infect Dis. 2005;11:844–50.
9. Zetola N, Francis JS, Nuermberger EL, Bishai WR. Community-acquired methicillin-resistant *Staphylococcus aureus:* an emerging threat. Lancet Infect Dis. 2005;5:275–86.
10. Styers D, Sheehan DJ, Hogan P, Sahm DF. Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus:* 2005 status in the United States. Ann Clin Microbiol Antimicrob. 2006;5:2.
11. Marshall C, Wesselingh S, McDonald M, Spelman D. Control of endemic MRSA-what is the evidence? A personal view. J Hosp Infect. 2004;56:253–68.
12. Salgado CD, Farr BM, Calfee DP. Community-acquired methicillin-resistant *Staphylococcus aureus:* a meta-analysis of prevalence and risk factors. Clin Infect Dis. 2003;36:131–9.
13. Sa-Leao R, Sanches IS, Couto I, Alves CR, de Lencastre H. Low prevalence of methicillin-resistant strains among *Staphylococcus aureus* colonizing young and healthy members of the community in Portugal. Microb Drug Resist. 2001;7:237–45.
14. Abudu L, Blair I, Fraise A, Cheng KK. Methicillin-resistant *Staphylococcus aureus* (MRSA): a community-based prevalence survey. Epidemiol Infect. 2001;126:351–6.
15. Nulens E, Gould I, MacKenzie F, Deplano A, Cookson B, Alp E, et al. *Staphylococcus aureus* carriage among participants at the 13th European Congress of Clinical Microbiology and Infectious Diseases. Eur J Clin Microbiol Infect Dis. 2005;24:145–8.
16. Weese JS. Methicillin-resistant *Staphylococcus aureus* in horses and horse personnel. Vet Clin North Am Equine Pract. 2004;20:601–13.
17. Duquette RA, Nuttall TJ. Methicillin-resistant *Staphylococcus aureus* in dogs and cats: an emerging problem? J Small Anim Pract. 2004;45:591–7.
18. Weese JS. Methicillin-resistant *Staphylococcus aureus:* an emerging pathogen in small animals. J Am Anim Hosp Assoc. 2005;41:150–7.
19. Lee JH. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appl Environ Microbiol. 2003;69:6489–94.
20. Simoons-Smit AM, Savelkoul PH, Stoof J, Starink TM, Vandenbroucke-Grauls CM. Transmission of *Staphylococcus aureus* between humans and domestic animals in a household. Eur J Clin Microbiol Infect Dis. 2000;19:150–2.
21. Manian FA. Asymptomatic nasal carriage of mupirocin-resistant, methicillin-resistant *Staphylococcus aureus* (MRSA) in a pet dog associated with MRSA infection in household contacts. Clin Infect Dis. 2003;36:e26–8.
22. O'Mahony R, Abbott Y, Leonard FC, Markey BK, Quinn PJ, Pollock PJ, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from animals and veterinary personnel in Ireland. Vet Microbiol. 2005;109:285–96.
23. van Duijkeren E, Wolfhagen MJ, Box AT, Heck ME, Wannet WJ, Fluit AC. Human-to-dog transmission of methicillin-resistant *Staphylococcus aureus.* Emerg Infect Dis. 2004;10:2235–7.
24. Weese JS, Archambault M, Willey BM, Hearn P, Kreiswirth BN, Said-Salim B, et al. Methicillin-resistant *Staphylococcus aureus* in horses and horse personnel, 2000–2002. Emerg Infect Dis. 2005;11:430–5.
25. Weese JS, Rousseau J, Willey BM, Archambault M, McGeer A, Low DE. Methicillin-resistant *Staphylococcus aureus* in horses at a veterinary teaching hospital: frequency, characterization, and association with clinical disease. J Vet Intern Med. 2006;20:182–6.

26. Weese JS, Caldwell F, Willey BM, Kreiswirth BN, McGeer A, Rousseau J, et al. An outbreak of methicillin-resistant *Staphylococcus aureus* skin infections resulting from horse to human transmission in a veterinary hospital. Vet Microbiol. 2006;114:160–4.
27. Weese JS, Rousseau J, Traub-Dargatz JL, Willey BM, McGeer AJ, Low DE. Community-associated methicillin-resistant *Staphylococcus aureus* in horses and humans who work with horses. J Am Vet Med Assoc. 2005;226:580–3.
28. Weese JS, Dick H, Willey BM, McGeer A, Kreiswirth BN, Innis B, et al. Suspected transmission of methicillin-resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. Vet Microbiol. 2006;115:148–55.
29. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Wayne (PA): The Committee; 2000.
30. Rankin S, Roberts S, O'Shea K, Maloney D, Lorenzo M, Benson CE. Panton-Valentine leukocidin (PVL) toxin positive MRSA strains isolated from companion animals. Vet Microbiol. 2005;108:145–8.
31. Simor AE, Ofner-Agostini M, Gravel D, Varia M, Paton S, McGeer A. Surveillance for methicillin-resistant *Staphylococcus aureus* in Canadian hospitals–a report update from the Canadian Nosocomial Infection Surveillance Program. Can Commun Dis Rep. 2005;31:33–40.
32. Leman R, Alvarado-Ramy F, Pocock S, Barg N, Kellum M, McAllister S, et al. Nasal carriage of methicillin-resistant *Staphylococcus aureus* in an American Indian population. Infect Control Hosp Epidemiol. 2004;25:121–5.
33. Simor AE, Ofner-Agostini M, Bryce E, McGeer A, Paton S, Mulvey MR. Laboratory characterization of methicillin-resistant *Staphylococcus aureus* in Canadian hospitals: results of 5 years of national surveillance, 1995–1999. J Infect Dis. 2002;186:652–60.
34. Simor AE, Ofner-Agostini M, Paton S, McGeer A, Loeb M, Bryce E, et al. Clinical and epidemiologic features of methicillin-resistant *Staphylococcus aureus* in elderly hospitalized patients. Infect Control Hosp Epidemiol. 2005;26:838–41.
35. Kourbatova EV, Halvosa JS, King MD, Ray SM, White N, Blumberg HM. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA 300 clone as a cause of health care-associated infections among patients with prosthetic joint infections. Am J Infect Control. 2005;33:385–91.
36. King MD, Humphrey BJ, Wang YF, Kourbatova EV, Ray SM, Blumberg HM. Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* USA 300 clone as the predominant cause of skin and soft-tissue infections. Ann Intern Med. 2006;144:309–17.
37. Johnson LB, Saeed S, Pawlak J, Manzor O, Saravolatz LD. Clinical and laboratory features of community-associated methicillin-resistant *Staphylococcus aureus*: is it really new? Infect Control Hosp Epidemiol. 2006;27:133–8.
38. Baptiste KE, Williams K, Willams NJ, Wattret A, Clegg PD, Dawson S, et al. Methicillin-resistant staphylococci in companion animals. Emerg Infect Dis. 2005;11:1942–4.
39. Eveillard M, Martin Y, Hidri N, Boussougant Y, Joly-Guillou ML. Carriage of methicillin-resistant *Staphylococcus aureus* among hospital employees: prevalence, duration, and transmission to households. Infect Control Hosp Epidemiol. 2004;25:114–20.

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Methicillin-resistant *Staphylococcus aureus* in Veterinary Doctors and Students, the Netherlands

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The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in the Netherlands, at 1.0%, is among the lowest in Europe. In 2004, a relationship between pig farming and a high risk for MRSA carriage was found. To investigate if those in professional contact with livestock are at higher risk for MRSA carriage, we screened 80 veterinary students and 99 veterinarians and questioned them about animal contacts and known MRSA risk factors. Of these, 27 students who did not have livestock contact were excluded from further analysis. We found 7 carriers of MRSA, a prevalence of 4.6%, which is similar to that found in patients who had previously been treated at foreign hospitals. A correlation of MRSA carriage with a specific animal group could not be established. To preserve the low prevalence of MRSA in the Netherlands, persons involved in the care of livestock should be isolated and screened on admission to the hospital.

In the Netherlands, the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical isolates of *S. aureus* has been <1% during the past decade (*1*,*2*) and, at 1.0%, remains one of the lowest in Europe (*3*). This low prevalence is best explained by the national "search and destroy" policy, which demands isolation and screening of patients at risk for MRSA carriage on admission to healthcare facilities. So far, the patients at risk have mainly been persons who had previously been admitted to or treated in foreign hospitals.

In 2004, three patients in our hospital who had no relation to foreign hospitals or exposure to other known sources of MRSA were unexpectedly found to carry MRSA. The patients were a pig farmer, a pig farmer's child, and a veterinarian's child. A subsequent screening of local pig farmers showed MRSA prevalence of >20%, which suggested that contact with pigs, at least in that region of the Netherlands, posed a substantial risk of acquiring MRSA (*4*). If that hypothesis were true, isolation on admission and screening of pig farmers and their family members for MRSA would be indicated. To further investigate if those in professional contact with livestock are at higher risk for MRSA carriage, we screened a selection of veterinary doctors and students.

Materials and Methods

We screened 80 veterinary students in the last phases of their education and 99 veterinarians attending a conference on livestock. Cultures were taken from both anterior nares and throat. All participants were asked to fill in a questionnaire about the type of animal contacts and possible exposure to known MRSA risk factors.

We incubated all cultures in a salt-enriched nutrient broth and after 24 hours subcultured them on blood agar plates and MRSA-ID agars (bioMerieux, La Balme Les Grottes, France). Colony morphology and latex agglutination test (Staphaurex, Remel, Lenexa, KS, USA) initially identified staphylococci; cefoxitine-disc diffusion determined methicillin resistance, according to Clinical and Laboratory Standards Institute criteria (*5*). All cefoxitine-resistant isolates underwent further identification and susceptibility testing to cefoxitine, gentamicin, vancomycin, teicoplanin, clindamycin, erythromycin, rifampicin, ciprofloxacin, cotrimoxazol, and tetracycline, using the Phoenix Automated Microbiology System (Becton

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Dickinson, Franklin Lakes, NJ, USA). We also performed *mecA* gene PCR, typing by pulsed-field gel electrophoresis (PFGE) with *Sma*I (the standard method), and *spa*-typing on all cefoxitine-resistant strains.

Results

The main characteristics of the veterinary doctors and students are listed in Table 1. Among the 179 persons tested, 7 (3.9%) MRSA carriers were found: 2 students and 5 veterinarians (Table 2). MRSA carriage varied depending on whether or not study participants had contact with livestock. MRSA carriage was 4.6% among 152 students and doctors in contact with livestock and 0% among 27 students who reported no contact with livestock. All MRSA carriers in this study had recent or regular contact with pigs and cows; only 3 veterinarians reported regular contact with sheep. Because all carriers reported contact with cows and pigs, no relative risk could be calculated (Table 3). In each group, 1 person indicated a known risk factor for MRSA carriage (1 had been admitted to a foreign hospital; 1 had an MRSA-positive family member), but both tested MRSA negative.

In addition to 7 MRSA isolates, *S. sciuri* was isolated from 1 veterinarian. This strain showed green colonies on the ID-MRSA plates and was Staphaurex positive (www.dcss.cs.amedd.army.mil/field/FLIP30/documents/pdfs/staphaurex_insert.pdf), which caused the risk to be wrongly identified as MRSA.

All cefoxitine-resistant isolates were susceptible to vancomycin, teicoplanin, rifampicin, and ciprofloxacin, but all were resistant to tetracycline. All MRSA strains and the *S. sciuri* were *mecA* positive and were resistant to digestion with restriction endonuclease *Sma*I when typing by PFGE was attempted, similar to the strains described by Voss et al. (*4*). Overall, 3 different MRSA types were identified by *spa* typing; 2 students and 1 veterinarian carried *spa*-type t011, 3 veterinarians carried *spa*-type t108, and 1 veterinarian carried *spa*-type t034. In contrast to the study of Voss et al., t108 was not a dominant *spa*-type.

Discussion

MRSA has been found in various animals, such as horses (*6*) and livestock (*7*), including pigs (*4*,*8*). So far, only 1 study has indicated transmission from livestock to caretakers (*4*). The extent of this transmission and its clinical significance remain unknown, also undetermined is whether persons in professions other than farming are at increased risk of becoming MRSA carriers. The overall MRSA prevalence in veterinary students and doctors involved in farm animal health in the Netherlands was about 160× higher than that among patients at hospital admissions (4.6% vs. 0.03%) (*9*); this prevalence falls within the range of that found in patients from foreign hospitals (3.5%–5%) (*10*). At least with regard to the search and destroy policy in the Netherlands, veterinarians and veterinary students who come in contact with the healthcare system may there-

Table 1. Main characteristics of veterinary students and veterinarians, the Netherlands

Characteristics	Veterinary students, n = 80, no. (%)	Veterinarians, n = 99, no. (%)
Mean age (range), y	26 (23–41)	43 (27–60)
Male	24 (30)	83 (83)
Professional contact limited to livestock	49 (63)	72 (73)
Professional contact limited to companion animals	27 (32)	0
Professional contact with livestock and companion animals	4 (5)	27 (27)
Contact with cows	48 (60)*	83 (83)†
Contact with pigs	37 (47)	72 (72)†
Contact with sheep	Not known	36 (36)†
Contact with pets at home	52 (65)	81 (81)
Risk factors for MRSA carriage‡	1 (1.2)	1 (1)

*Regular contact in past 3 months.
†Regular part of practice and/or regular contact in the past 6 months.
‡MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 2. Characteristics and type of animal contact of MRSA carriers, the Netherlands*

Case	Sex	Profession	Pigs	Cows	Horses	Sheep	Companion animals
1	F	Student	X	X		?	
2	F	Student	X	X	X	?	
3	M	Veterinarian	X	X	X	X	X
4	M	Veterinarian	X	X	X		X
5	M	Veterinarian	X	X		X	
6	M	Veterinarian	X	X		X	
7	M	Veterinarian	X	X			

*MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 3. Estimates of relative risk for exposure to types of animals for veterinary students and veterinarians, the Netherlands

Type of animal	Relative risk	95% Confidence interval
Pigs*	9.0	0.52–154
Cows*	5.3	0.31–90
Sheep†	4.35	0.52–40
Companion animals	0.86	0.17–4.2
Horses	0.72	0.14–3.6

*The number of carriers without exposure in this group was 0; estimate of relative risk was made by adding 0.5 to all groups.
†Data on veterinarians only.

fore qualify as patients at high risk, warranting screening and isolation on admission to hospitals.

The high frequency of MRSA carriage among veterinary doctors and students is unexpected. While protective coveralls and boots are routinely used during veterinary contact with livestock, protective masks are not. Because *S. aureus* colonization and transmission occur mainly through contact from the hands to the anterior nares, the standard measures are probably insufficient to prevent MRSA colonization. Therefore, masks and gloves could be considered as additional protective measures.

Although low in comparison with several other countries, the quantity and intensity of antimicrobial use in livestock has increased in the Netherlands (*11*). Data from 1997 to 2004 show that the main antimicrobial classes used in livestock are tetracycline and trimethoprim sulfonamide combinations. All the MRSA strains in this study, and all the strains found by Voss et al., were resistant to tetracycline.

We conclude that veterinary doctors and students caring for livestock have a high risk of being colonized by MRSA. The percentage of MRSA carriage in the doctors and students surveyed is such that, to preserve the low prevalence of MRSA in the Netherlands, all persons involved in the care of livestock should be isolated and screened on admission to the hospital, according to national policy. Further investigation is needed to determine the exact source of MRSA in livestock and the effect of risk factors such as the use of antimicrobial agents on MRSA carriage in livestock. This type of research should be conducted in other countries to find out if this phenomenon is limited to the Netherlands or is international.

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References

1. Tiemersma EW, Bronzwaer SL, Degener JE, Lyytikäinen O, Schrijnemakers P, Bruinsma N, et al. Methicillin-resistant *Staphylococcus aureus* in Europe, 1999–2002. Emerg Infect Dis. 2004;10:1627–34.
2. Voss A, Milatovic D, Wallrauch-Schwarz C, Rosdahl VT, Braveny I. Methicillin-resistant *Staphylococcus aureus* in Europe. Europ J Clin Microbiol Infect Dis. 1994;13:50–5.
3. European Antimicrobal Resistance Surveillance System (EARSS). EARSS annual report 2004. [cited 2006 Oct 4]. Available from http://www.rivm.nl/earss/Images/EARSS%20annual%20report%202004%20webversie_tcm61-25345.pdf.
4. Voss A, Loeffen F, Bakker J, Wulf M, Klaassen C. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg Infect Dis. 2005;11:1965–6.
5. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI/NCCLS M100-S15. Wayne (PA): The Institute; 2005.
6. Weese JS, Archambault M, Willey BM, Dick H, Hearn P, Kreiswirth BN, et al. Methicillin-resistant *Staphylococcus aureus* in horses and horse personnel, 2000–2002. Emerg Infect Dis. 2005;11:430–5.
7. Lee JH. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appl Environ Microbiol. 2003;69:6489–94.
8. Armand-Lefevre L, Ruimy R, Andremont A. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg Infect Dis. 2005;11:711–4.
9. Wertheim HF, Vos MC, Boelens HA, Voss A, Vandenbroucke-Grauls CM, Meester MH, et al. Low prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) at hospital admission in the Netherlands: the value of search and destroy and restrictive antibiotic use. J Hosp Infect. 2004;56:321–5.
10. Kaiser AM, Schultsz C, Kruithof GJ, Debets-Ossenkopp YJ, Vandenbroucke-Grauls CM. Carriage of resistant micro-organisms in repatriates from foreign hospitals to the Netherlands. Clin Microbiol Infect. 2004;110:972–9.
11. Mevius DJ, Pellicaan C, van Pelt W. MARAN-2004: monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2004. [cited 2006 Oct 6]. Available from http://www.cidc-lelystad.wur.nl/NR/rdonlyres/7F79ACE6-0FD2-41AB-81B2-BB17FA89603C/11382/MARAN2004web1.pdf

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Evaluating Detection of an Inhalational Anthrax Outbreak

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Timely detection of an inhalational anthrax outbreak is critical for clinical and public health management. Syndromic surveillance has received considerable investment, but little is known about how it will perform relative to routine clinical case finding for detection of an inhalational anthrax outbreak. We conducted a simulation study to compare clinical case finding with syndromic surveillance for detection of an outbreak of inhalational anthrax. After simulated release of 1 kg of anthrax spores, the proportion of outbreaks detected first by syndromic surveillance was 0.59 at a specificity of 0.9 and 0.28 at a specificity of 0.975. The mean detection benefit of syndromic surveillance was 1.0 day at a specificity of 0.9 and 0.32 days at a specificity of 0.975. When syndromic surveillance was sufficiently sensitive to detect a substantial proportion of outbreaks before clinical case finding, it generated frequent false alarms.

In the early stage of an inhalational anthrax outbreak, a 1-day delay in the initiation of chemoprophylaxis and treatment of exposed persons can result in thousands of additional deaths and millions of dollars of additional expenditures (*1*,*2*). Thus, timely detection of an inhalational anthrax outbreak is critical. Rapid detection is also important for disease outbreaks that result from other bioterrorism agents and from emerging infectious diseases, such as severe acute respiratory syndrome or avian influenza (*3*).

To detect an epidemic such as inhalational anthrax, which is nonendemic and results in severe symptoms, public health authorities have relied traditionally on identification and rapid reporting of the sentinel clinical case. However, because the perceived likelihood of a bioterrorism attack has increased, public health authorities have sought novel approaches for rapid outbreak detection. One approach that has received considerable economic investment over the past 5 years is syndromic surveillance. This approach follows prediagnostic data sources in an attempt to detect an increase in the prevalence of nonspecific symptoms. For example, the BioSense system (*4*), developed by the Centers for Disease Control and Prevention (CDC) at a cost of >$75 million (*5*), follows records of outpatient visits, pharmaceutical prescriptions, and laboratory orders in an attempt to detect disease outbreaks rapidly. Hundreds of similar systems are maintained or are under development by various groups around the world (*6*). Other examples include systems operated by the Department of Homeland Security (*5*) and academic centers in partnership with state or county public health departments (*7*–*9*).

In addition to supporting outbreak detection, these syndromic surveillance systems provide situational awareness for public health authorities and may serve other purposes. Nevertheless, a major justification for these systems is outbreak detection. Despite substantial investment in syndromic surveillance and calls for further research from groups such as the Institute of Medicine (*3*), little evidence exists to suggest how syndromic surveillance will perform relative to clinical case finding for detection of an inhalational anthrax outbreak (*10*). The reason for this lack of evidence is that data from real outbreaks are not available to evaluate the performance of syndromic surveillance alone or in comparison to clinical case finding. Moreover, even if data were available from a large-scale outbreak, those data would allow only an evaluation of performance in 1 specific setting. CDC recently endorsed the use of simulated outbreaks to address the dearth of outbreak data (*11*), but existing simulation studies have not compared detection through clinical case finding with syndromic surveillance (*12*–*14*). Our aim was to develop a model for simulating use of healthcare services after a large-scale exposure to aerosol anthrax spores and then to use this

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model to estimate the detection benefit of syndromic surveillance compared with clinical case finding.

Methods

Study Design

We developed a model to simulate the dispersion of released anthrax spores; the infection of exposed persons; the progression of disease in infected persons; and symptomatic persons' use of the healthcare system, including blood culture testing in clinical settings. Using the simulation model, we generated outbreak signals and time until the first clinical diagnosis for 3 amounts of spores released. To incorporate into the model the uncertainty in parameter values, we used a Latin hypercube sampling design, which allows many parameter values to vary simultaneously (*15*). The 3,000 simulated signals generated with this sampling strategy were superimposed in turn onto baseline administrative records of ambulatory healthcare visits in the Norfolk, Virginia, area. These records are generated daily and similar types of records are used widely for syndromic surveillance (*4*,*7*,*9*). We assessed the usefulness of syndromic surveillance by modeling the healthcare system use that would occur after an anthrax attack and superimposing this use onto actual administrative data over 1-year period. Finally, we assessed, over a range of specificity, the sensitivity and timeliness of syndromic surveillance and the detection benefit of syndromic surveillance compared with clinical case finding for each simulated outbreak. We summarize our methods in the remainder of this section and refer readers to the online Technical Appendix (available from http://www.cdc.gov/ncidod/EID/vol12no12/06-0331_app.htm) for additional details.

Simulation Model

The simulation model builds on our previous work (*16*–*18*) and is composed of 4 components: dispersion, infection, disease, and healthcare system use. The dispersion model simulates the number of anthrax spores a person would inhale at locations throughout the region after release of aerosolized spores. We used the Hazard Prediction and Assessment Capability (HPAC) software developed by the Defense Threat Reduction Agency to simulate the dispersion of spores (*19*). The HPAC model accounts for factors such as atmospheric conditions and terrain. We simulated a point release of 3 amounts of anthrax spores: 1 kg, 0.1 kg, and 0.01 kg (Figure 1A).

The infection model simulates the number of persons infected, according to residential address and dispersion of spores (Figure 1B). The probability of infection given exposure to an amount of spores was modeled by using a probit regression model. The disease model uses a semi-Markov process to simulate the progression of infected persons through 3 discrete states of disease. Each infected person began in the incubation state and then progressed through the prodromal state and the fulminant state. The time in each state was sampled from a log normal distribution.

The healthcare use model uses a semi-Markov process to simulate the probability and timing of a symptomatic person seeking care and submission of blood for culture and culture results when care is sought. For persons in the prodromal or fulminant state of disease who sought care, the instantaneous probability of seeking care increased linearly over the duration of the state. For patients whose blood samples were cultured, the testing process was modeled as the transition through 2 discrete states: growth and isolation. The time spent in each of these states was modeled by using an exponential distribution.

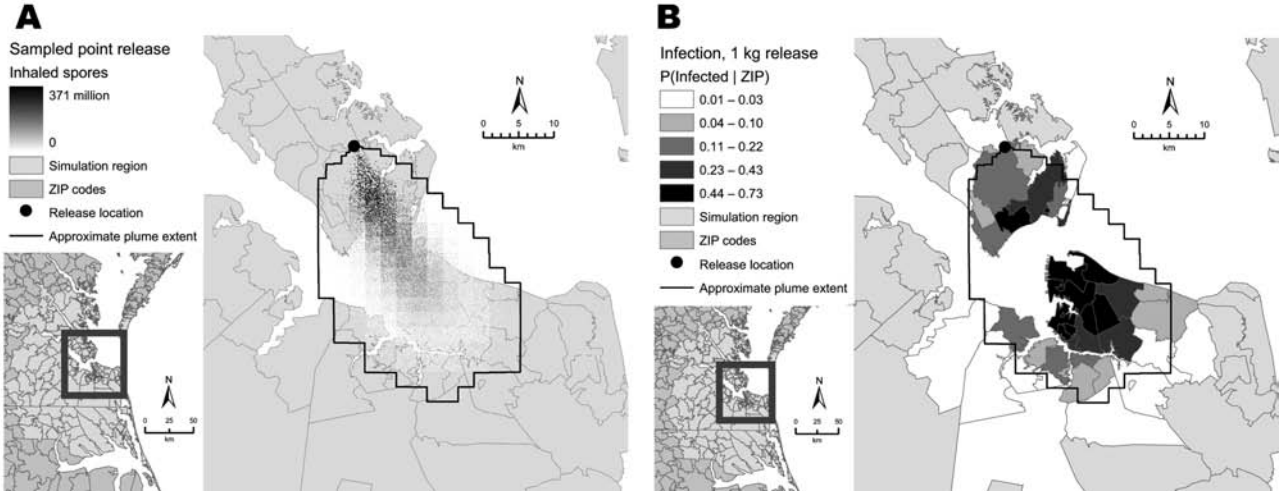

Figure 1. Maps showing output from dispersion (A) and infection (B) components of the simulation model. The dispersion component simulates geographic distribution of anthrax spores after an aerosol release. The infection component simulates infection of persons exposed to spores.

Data for Simulation Model

The infection model used an infection function corresponding to the data reported by Glassman (*20*). This is a probit model with a 50% lethal dose (LD_{50}) of 8,600 spores and a slope of 0.67. Uncertainty exists about the values for many of the parameters in the disease and healthcare use models. To incorporate this uncertainty into our estimates, we used a Latin hypercube sampling approach to sample parameter values for random variables in our simulation model (*15*). This approach requires specifying equal probability bins for parameter values. We specified 3 bins for each parameter value, a narrow bin around the most likely estimate, and wider bins on either side of the estimate. Table 1 shows the bins we used for each parameter value, the probability distribution that each value parameterizes, and the sources that we used to define the bins.

We used previous work modeling anthrax for the distribution of time periods in each disease state (*2*,*21*,*22*). For the probability of seeking care while in the prodromal disease state, cross-sectional surveys indicate that 14%–30% of persons visit a physician at some point during an episode of upper respiratory tract illness (*23*,*24*). For the fulminant disease state, we estimated the probability of seeking care before death as 90%–95%, given the severity of the symptoms in that state.

After a person made a healthcare visit, we simulated the syndrome assigned to the person by using probabilities that reflect the distribution of clinical presentations for inhalational anthrax reported in the literature (*25*,*26*). Because we considered only respiratory syndromes for surveillance, we varied directly only the probability of being assigned a respiratory syndrome to persons in the prodromal disease state.

For visits from persons in either symptomatic disease state, the estimate of sensitivity from published studies of blood culture testing was 0.8–0.9 (*27*). For a visit in the prodromal state, we estimated the probability of a physician ordering a blood culture as 0.01–0.015 on the basis of data from the National Ambulatory Medical Care Survey (*28*). For a visit in the fulminant state of disease, we estimated the probability of a blood culture test as 0.9–0.95. After gram-positive rods grew in the blood culture, we estimated the probability of isolating the organism to be 0.8–0.9 (*29*). We modeled the time until growth and isolation as exponential (*25*,*30*).

Baseline Data and Release Scenarios

We used records of ambulatory visits in the Norfolk, Virginia, region acquired from the TRICARE health maintenance organization as a baseline onto which we superimposed simulated outbreak records. The data covered the period 2001–2003, and the simulation region included 17 clinical facilities within an ≈160-km × 200-km area that encompasses 158 ZIP codes from 2 states. Over the 3 years of available data, 427,634 persons made >5 million visits. We classified the records into syndromes by using the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) to syndrome mapping defined by the ESSENCE system (*7*) and used only 351,749 records for which persons were classified as having a respiratory syndrome. The Human Subjects Panel at the Stanford School of Medicine approved the use of these data for this study. We examined 3 scenarios defined by the amount of spores released: 1 kg, 0.1 kg, and 0.01 kg. For each scenario, we performed 1,000 simulations.

Table 1. Sampling intervals for parameter values in the simulation model*

Parameter	Parameter value intervals	Probability distribution	Source†
Disease model			
Incubation duration, d; median	(5, 9) (9, 11) (11, 15)	Log normal	(*2*,*21*,*22*)
Incubation duration, dispersion	(1.5, 1.9) (1.9, 2.1) (2.1, 2.5)	Log normal	(*2*,*21*,*22*)
Prodromal duration, d; median	(1.5, 2.3) (2.3 ,2.7) (2.7, 3.5)	Log normal	(*2*,*22*)
Prodromal duration, dispersion	(1.2, 1.4) (1.4, 1.5) (1.5, 1.7)	Log normal	(*2*,*22*)
Healthcare use			
Probability of visit, prodromal state	(0.05, 0.25) (0.25, 0.35) (0.35, 0.55)	Bernoulli	(*23*,*24*)
Probability of visit, fulminant state	(0.7, 0.9) (0.9, 0.95) (0.95, 1)	Bernoulli	Estimate
Probability of respiratory syndrome, prodromal state	(0.5, 0.7) (0.7, 0.8) (0. 8,1)	Bernoulli	(*25*,*26*)
Blood culture test, prodromal state	(0.001, 0.01) (0.01, 0.015) (0.015, 0.025)	Bernoulli	(*28*)
Blood culture test, fulminant state	(0.7, 0.9) (0.9, 0.95) (0.95, 1)	Bernoulli	Estimate
Sensitivity of blood culture	(0.5, 0.8) (0.8, 0.9) (0.9, 1)	Bernoulli	(*27*)
Time until blood culture growth, d	(0.4, 0.8) (0.8, 1.0) (1.0, 1.4)	Exponential	(*30*)
Probability of isolation given growth	(0.5, 0.8) (0.8, 0.9) (0.9, 1)	Bernoulli	(*29*)
Time until blood culture isolation, d	(0.5, 0.6) (0.6, 0.9) (0.9, 1.5)	Exponential	(*25*)

*Using a Latin hypercube strategy, a value for each parameter was sampled by randomly selecting 1 of the 3 intervals for the parameter and randomly sampling a value on the selected interval. The sampled values parameterize probability distributions, which are sampled for the simulation model.
†References that support the parameter value intervals.

Outbreak Detection

The time to outbreak detection through clinical case finding for a simulated outbreak was calculated for each simulated outbreak as the time between exposure to spores and the first positive blood culture. To calculate time to outbreak detection through syndromic surveillance, we superimposed the simulated records for respiratory syndrome visits onto the authentic baseline data, beginning on a randomly selected date in 2003, and then applied the outbreak detection algorithm to the combined baseline and simulated data. The outbreak detection algorithm used a time-series model (*31*) to generate daily 1-step-ahead forecasts for the total number of respiratory syndrome visits (*13*) and then applied a cumulative sum (*32*) to the forecast residual. To vary the specificity of the detection algorithm, we varied the decision threshold of the cumulative sum.

Evaluation Metrics

To evaluate outbreak detection through syndromic surveillance, we calculated sensitivity, specificity, and timeliness at a range of decision thresholds. Timeliness is the duration between the release of anthrax spores and the first report of an outbreak. We also computed the detection benefit of syndromic surveillance relative to clinical case finding, and the proportion of runs with a detection benefit >0. Detection benefit is the potential time saved in detection from using syndromic surveillance compared with clinical case finding. The benefit is calculated as the difference in the timeliness between syndromic surveillance and clinical case finding in those simulations in which detection occurred first by syndromic surveillance. When an outbreak was not detected by syndromic surveillance, the detection benefit was 0. For a given release scenario, each of the 1,000 simulations integrated both randomness in the component model outputs as well as uncertainty in component model parameters. Each of the 1,000 simulations is a sample from the integrated distribution of possible outcomes. To indicate the spread of the integrated uncertainty distribution, we calculated the upper and lower deciles from the 1,000 simulations. For plots, we calculated 95% confidence intervals, which reflect finiteness of the simulation.

Results

Detection Performance of Clinical Case Finding

Because all outbreaks were ultimately detected by clinical case finding through routine blood culture, the sensitivity of this approach was 1.0 for the scenarios considered. Clinical case finding detected outbreaks from an average of 3.7 days to 4.1 days after release, with larger amounts of spores detected before smaller amounts (Table 2). Results from analyses of additional release scenarios (data not shown) suggested that the influence of amount released on time to detection was mediated, in part, through the number infected. Mean timeliness across the scenarios examined was associated with the mean number infected (Pearson's r –0.94, 95% confidence interval –0.98 to –0.79), and an increase of 10,000 infected persons resulted in a decrease in the time until detection of ≈4 hours.

Table 2. Average numbers of persons infected and average times to outbreak detection through clinical case finding for 3 release scenarios*

Amount released (kg)	Mean no. infected	Mean days to detection
1	49,000	3.7 (2.5, 5.0)
0.1	31,000	3.9 (2.7, 5.3)
0.01	15,000	4.1 (2.9, 5.5)

*Values in parentheses are 10th and 90th percentiles of the distribution.

Detection Performance of Syndromic Surveillance

The sensitivity and timeliness of syndromic surveillance were influenced by the release amount and by specificity. Table 3 shows this relationship over the release scenarios examined and 2 levels of specificity. At a specificity of 0.90, a 1-kg release was detected in 100% of our simulations (sensitivity 1.0) at a mean detection time of 3.1 days. For a release that was much smaller, 0.01 kg, sensitivity was 0.94, and the mean detection time increased to 3.6 days. Although the sensitivity of syndromic surveillance was high when we set specificity to 0.90, this specificity resulted in a false alarm (false-positive detection) ≈1 every 10 days. By increasing specificity to 0.975, we reduced the false alarm rate to ≈1 every 40 days (Table 3). However, with increased specificity, the sensitivity of syndromic surveillance decreased (from 0.98 to 0.82 depending on the size of the release) and the mean time until detection lengthened to 4.3 days for a 1-kg release and to 5.1 days for a 0.01-kg release (Table 3).

Results from analyses of additional release scenarios (data not shown) indicated that the trends in sensitivity and timeliness across release amount were mediated to some extent by the number infected. Sensitivity was a nonlinear function of the number of persons infected, with sensitivity increasing more quickly when fewer persons were infected. At a specificity of 0.975, an increase of 10,000 infected persons resulted in a decrease in time to detection of ≈6 hours.

Detection Benefit of Syndromic Surveillance Compared with Clinical Case Finding

The detection benefit of syndromic surveillance compared with clinical case finding was influenced by specificity and the release amount. Table 3 shows this relationship for the release amounts examined and 2 levels

Table 3. Sensitivity, time to outbreak detection (timeliness), proportion of outbreaks detected through syndromic surveillance before clinical case finding, and mean detection benefit of syndromic surveillance compared with clinical case finding for 3 release scenarios and 2 levels of specificity*

Amount released (kg)	Specificity 0.900 (1 false alarm every 10 d)				Specificity 0.975 (1 false alarm every 40 d)			
	Sensitivity per outbreak	Mean timeliness, d	Proportion with detection benefit	Mean detection benefit, d	Sensitivity per outbreak	Mean timeliness, d	Proportion with detection benefit	Mean detection benefit, d
1	1.00	3.1 (0, 5)	0.59	1.0 (0, 3.3)	0.98	4.3 (2, 7)	0.28	0.32 (0, 1.0)
0.1	0.99	3.3 (0, 6)	0.55	1.0 (0, 3.5)	0.95	4.7 (2, 7)	0.24	0.33 (0, 1.1)
0.01	0.94	3.6 (0, 7)	0.51	1.1 (0, 3.7)	0.82	5.1 (2, 8)	0.19	0.33 (0, 1.3)

*Values in parentheses are 10th and 90th percentiles of the distribution.

of specificity. When the specificity was 0.9, syndromic surveillance detected from 51% to 59% of outbreaks before clinical case finding, and the mean detection benefit was 1.0–1.1 days, but this specificity resulted in a false alarm every 10 days. At a specificity of 0.975, which reduced false alarms to 1 every 40 days, syndromic surveillance detected 19%–28% of outbreaks before clinical case finding and the mean detection benefit was 0.32–0.33 days, or ≈8 hours. Figure 2 shows that for the 0.01-kg and 1-kg release scenarios (results for the 0.1-kg release are similar, but are not shown), the proportion of outbreaks detected first by syndromic surveillance and the mean detection benefit of surveillance each increased as specificity decreased. Figure 2 also shows that the release amount had a strong effect on the proportion of outbreaks detected first by syndromic surveillance but that it did not have a strong effect on the mean detection benefit.

At a set specificity, syndromic surveillance tended to detect a higher proportion of outbreaks before clinical case finding with increasing release amount. The mean detection benefit, in contrast, tended to decrease when the amount of spores released increased. This decrease in average detection benefit occurred because even though syndromic surveillance detected more outbreaks before clinical case finding as the release amount increased, the detection benefit for the additional outbreaks was small, and the average detection benefit thus decreased.

Discussion

When we compared the performance of clinical case finding with that of syndromic surveillance for detecting an inhalational anthrax outbreak, we found that clinical case finding detected outbreaks on average 3.7–4.6 days after release of spores. The ability of syndromic surveillance to detect an outbreak before clinical case finding was influenced by both specificity and release size, with specificity being the predominant factor. Our results suggest that syndromic surveillance could detect an inhalational anthrax outbreak before clinical case finding. However, we regularly observed a detection benefit only when syndromic surveillance operated at a specificity in the range of 0.9, which corresponds to 1 false alarm every 10 days. When operating at this relatively low specificity with a concomitant high sensitivity, syndromic surveillance detected outbreaks, on average, 1 day before clinical case finding did.

One of the most useful findings of our study was the tradeoff between sensitivity and specificity of syndromic surveillance. To reduce the false alarm rate, specificity must be high. However, as specificity increased in our study, the sensitivity of syndromic surveillance decreased, and the proportion of outbreaks that was detected first by syndromic surveillance decreased more substantially. If the response to a result from syndromic surveillance is resource intensive and includes follow-up investigations in multiple healthcare settings, then a false alarm rate of 1 every 10 days may be too high for such a system to be useful. Alternatively, if public health personnel can rule out false-positive results with minimal investment, then a higher rate of false alarms may be acceptable.

The detection benefit of syndromic surveillance might be an important lead, depending on the action triggered by a surveillance alarm. Because many clinical and public health departments have defined protocols for actions after clinical confirmation of an inhalational anthrax case (*33*), the action after detection of a clinical case is fairly well defined in many jurisdictions. In contrast, the appropriate action after a result from syndromic surveillance system is not well-defined (*34*). For example, some public health departments routinely wait 1 day for a second alarm before taking action (*35*). This strategy could eliminate the potential detection benefit of syndromic surveillance. Another concern is the relatively low specificity at which syndromic surveillance must operate to consistently result in a detection benefit. A system producing this many false alarms may result in excessive costs, and users may minimize the importance of these results.

To be useful, however, syndromic surveillance does not necessarily have to detect all outbreaks, or even most outbreaks, before a clinician detects the first case. The additional lead in detection offered by syndromic surveillance in some outbreaks may result in enough benefit to support the use of syndromic surveillance. Syndromic surveillance may also be useful for applications other than detecting an outbreak caused by bioterrorism; e.g., for detecting other types of disease outbreaks (*36*), for ruling out the existence

of an outbreak, or for evaluating the effect of a public health intervention. Assessment of the question of the utility of syndromic surveillance in general would require consideration of a broader range of costs and benefits than we included in our study.

Our methods are an advance over those used in previous studies because we were able to examine rigorously, within a single modeling framework, the ability of clinical case finding and syndromic surveillance to detect anthrax outbreaks. The nature of our model allowed us to vary some outbreak characteristics directly (e.g., release amount) and to incorporate the uncertainty in parameter values into our final estimates of detection performance and detection benefit. Although our sampling approach did allow us to vary many parameter values simultaneously, it did not clarify how the results vary in relation to changes in the value of a single parameter. Our estimate of detection performance through syndromic surveillance is comparable to estimates observed through studies that used simulation models (*12*,*37*), but those studies did not allow direct comparison of detection through syndromic surveillance with detection through clinical case finding. Our estimate of the time until detection through clinical case finding is longer than the estimate used by the authors of a study aimed at modeling response strategies to an anthrax outbreak (*2*), but those authors did not provide a clear rationale for the value they chose. An initial presumptive diagnosis may occur earlier than the first positive blood culture result (e.g., through clinical symptoms and chest radiographs), but a decision for large-scale intervention would likely not be made until at least after the first definitive diagnosis was made.

In our study, we considered 1 approach to syndromic surveillance for an outbreak resulting from 1 type of organism, and we considered clinical case finding through 1 type of routinely applied diagnostic test. There are many different approaches to syndromic surveillance; e.g., different types of data and different detection algorithms. Although different approaches to surveillance might produce different results, the choice of the infectious organism is likely to have a greater effect on results. Anthrax is relatively unique among bioterrorism agents in that a routinely used diagnostic test (i.e., blood culture) will identify the organism definitively. The benefit of syndromic surveillance relative to clinical case finding may therefore be greater for outbreaks caused by other organisms, and an anthrax outbreak may be a worst-case scenario for syndromic surveillance.

Syndromic surveillance detected an inhalation anthrax outbreak before the first clinical case was diagnosed in as many as half of simulated outbreaks. However, the potential detection benefit of syndromic surveillance compared with clinical case finding depended critically on the specificity and sensitivity at which a surveillance system operated and on the size of the outbreak. When syndromic surveillance was sufficiently sensitive to detect a substantial proportion of outbreaks, it generated frequent false alarms. Public health authorities should be aware that the

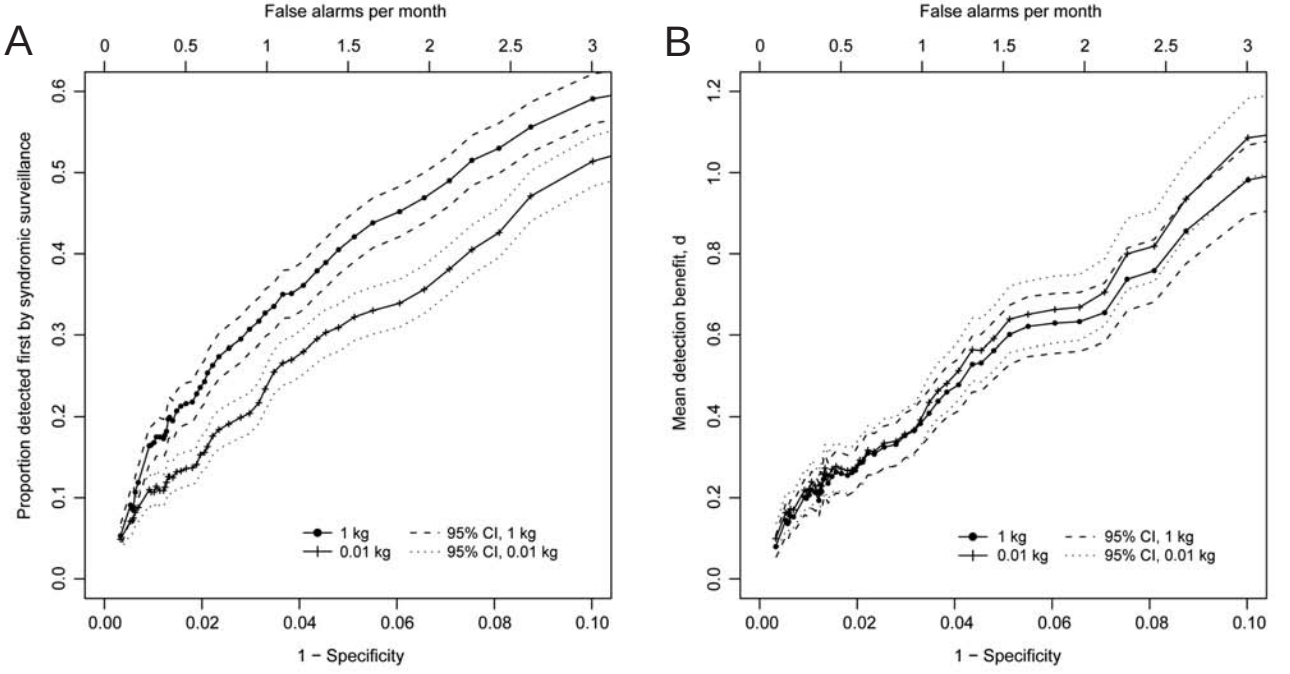

Figure 2. Proportion of inhalational anthrax outbreaks detected by syndromic surveillance before clinical case finding (A) and mean detection benefit of syndromic surveillance compared with clinical case finding as a function of specificity (and false-alarm rate)(B) for 3 release scenarios. CI, confidence interval.

potential detection benefit of syndromic surveillance compared with clinical case finding is influenced strongly by the specificity at which a surveillance system operates. To help detect outbreaks more rapidly, future research should examine the cost-effectiveness of syndromic surveillance and explore approaches to linking syndromic surveillance and clinical case finding more closely.

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References

1. Kaufmann AF, Meltzer MI, Schmid GP. The economic impact of a bioterrorist attack: are prevention and postattack intervention programs justifiable? Emerg Infect Dis. 1997;3:83–94.
2. Wein LM, Craft DL, Kaplan EH. Emergency response to an anthrax attack. Proc Natl Acad Sci U S A. 2003;100:4346–51.
3. Smolinski M, Hamburg ML. Lederberg J, editors. Microbial threats to health: emergence, detection, and response. Washington: National Academy Press; 2003.
4. Loonsk JW. BioSense: a national initiative for early detection and quantification of public health emergencies. MMWR Morb Mortal Wkly Rep. 2004;53(Suppl):53–5.
5. United States Government Accountability Office. Information technology. Federal agencies face challenges in implementing initiatives to improve public health infrastructure. Volume GAO-05–308. Washington: The Office; 2005.
6. Buehler JW, Berkelman RL, Hartley DM, Peters CJ. Syndromic surveillance and bioterrorism-related epidemics. Emerg Infect Dis. 2003;9:1197–204.
7. Lombardo J, Burkom H, Elbert E, Magruder S, Lewis SH, Loschen W, et al. A systems overview of the electronic surveillance system for the early notification of community-based epidemics (ESSENCE II). J Urban Health. 2003;80(Suppl 1):i32–42.
8. Wagner MM, Robinson JM, Tsui F-C, Espino JU, Hogan WR. Design of a national retail data monitor for public health surveillance. J Am Med Inform Assoc. 2003;10:409–18.
9. Yih WK, Caldwell B, Harmon R, Kleinman K, Lazarus R, Nelson A, et al. National Bioterrorism Syndromic Surveillance Demonstration Program. MMWR Morb Mortal Wkly Rep. 2004;53(Suppl):43–9.
10. Bravata DM, McDonald KM, Smith WM, Rydzak C, Szeto H, Buckeridge DL, et al. Systematic review: surveillance systems for early detection of bioterrorism-related diseases. Ann Intern Med. 2004;140:910–22.
11. Buehler JW, Hopkins RS, Overhage JM, Sosin DM, Tong V. Framework for evaluating public health surveillance systems for early detection of outbreaks: recommendations from the CDC Working Group. MMWR Recomm Rep. 2004;53:1–11.
12. Nordin JD, Goodman M, Kuldorff M, Ritzwoller DP, Abrams AM, Kleinman K, et al. Simulated anthrax attacks and syndromic surveillance. Emerg Infect Dis. 2005;11:1394–8.
13. Reis BY, Pagano M, Mandl KD. Using temporal context to improve biosurveillance. Proc Natl Acad Sci U S A. 2003;100:1961–5.
14. Stoto M, Schonlau M, Mariano L. Syndromic surveillance: is it worth the effort? Chance. 2004;17:19–24.
15. McKay M, Beckman R, Conover W. A comparison of three methods for selecting values of input variables in the analysis of output from a computer code. Technometrics. 1979;21:239–45.
16. Buckeridge DL. A method for evaluating outbreak detection in public health surveillance systems that use adminstrative data. [Doctoral dissertation]. Stanford (CA): Stanford University; 2005.
17. Buckeridge DL, Burkom H, Moore A, Pavlin J, Cutchis P, Hogan W. Evaluation of syndromic surveillance systems: design of an epidemic simulation model. MMWR Morb Mortal Wkly Rep. 2004;53(Suppl):137–43.
18. Buckeridge DL, Switzer P, Owens D, Siegrist D, Pavlin J, Musen M. An evaluation model for syndromic surveillance: assessing the performance of a temporal algorithm. MMWR Morb Mortal Wkly Rep. 2005;54(Suppl):109–15.
19. Defense Threat Reduction Agency. The HPAC user's guide. Hazard prediction and assessment capability, version 4.0. Fort Belvior (VA): The Agency; 2001.
20. Glassman HN. Industrial inhalation anthrax. Discussion. Bacteriol Rev. 1965;30:657–9.
21. Brookmeyer R, Blades N, Hugh-Jones M, Henderson DA. The statistical analysis of truncated data: application to the Sverdlovsk anthrax outbreak. Biostatistics. 2001;2:233–47.
22. Webb GF, Blaser MJ. Mailborne transmission of anthrax: modeling and implications. Proc Natl Acad Sci U S A. 2002;99:7027–32.
23. McIsaac WJ, Levine N, Goel V. Visits by adults to family physicians for the common cold. J Fam Pract. 1998;47:366–9.
24. Metzger KB, Hajat A, Crawford M, Mostashari F. How many illnesses does one emergency department visit represent? Using a population-based telephone survey to estimate the syndromic multiplier. MMWR Morb Mortal Wkly Rep. 2004;53(Suppl):106–11.
25. Inglesby TV, O'Toole T, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Anthrax as a biological weapon, 2002: updated recommendations for management. JAMA. 2002;287:2236–52.
26. Jernigan JA, Stephens DS, Ashford DA, Omenaca C, Topiel MS, Galbraith M, et al. Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States. Emerg Infect Dis. 2001;7:933–44.
27. Reimer LG, Wilson ML, Weinstein MP. Update on detection of bacteremia and fungemia. Clin Microbiol Rev. 1997;10:444–65.
28. National Center for Health Statistics. Public use data tapes: National Ambulatory Medical Care Survey 2003. Hyattsville (MD): The Center; 2003.
29. Begier EM, Barrett N, Mshar P, Johnson D, Hadler J; Connecticut Bioterrorism Field Epidemiology Response Team. Gram-positive rod surveillance for early anthrax detection. Emerg Infect Dis. 2005;11:1483–6.
30. Beekmann SE, Diekema DJ, Chapin KC, Doern GV. Effects of rapid detection of bloodstream infections on length of hospitalization and hospital charges. J Clin Microbiol. 2003;41:3119–25.
31. Box GEP, Jenkins GM. Time series analysis, forecasting and control. Englewood Cliffs (NJ): Prentice Hall; 1976.
32. Page E. Continuous inspection schemes. Biometrika. 1954;41:100–15.
33. Tengelsen L, Hudson R, Barnes S, Hahn C. Coordinated response to reports of possible anthrax contamination, Idaho, 2001. Emerg Infect Dis. 2002;8:1093–5.

34. Pavlin JA. Investigation of disease outbreaks detected by "syndromic" surveillance systems. J Urban Health. 2003;80(Suppl 1):i107–14.
35. Heffernan R, Mostashari F, Das D, Karpati A, Kuldorff M, Weiss D. Syndromic surveillance in public health practice, New York City. Emerg Infect Dis. 2004;10:858–64.
36. Marx MA, Rodriguez CV, Greenko J, Das D, Heffernan R, Karpati AM, et al. Diarrheal illness detected through syndromic surveillance after a massive power outage: New York City, August 2003. Am J Public Health. 2006;96:547–53.
37. Miller B, Kassenborg H, Dunsmuir W, Griffith J, Hadidi M, Nordin JD, et al. Syndromic surveillance for influenzalike illness in ambulatory care network. Emerg Infect Dis. 2004;10:1806–11.

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Spongiform Encephalopathy in a Miniature Zebu

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The first case of spongiform encephalopathy in a zebu (*Bos indicus*) was identified in a zoo in Switzerland. Although histopathologic and immunohistochemical analyses of the central nervous system indicated a diagnosis of bovine spongiform encephalopathy (BSE), molecular typing showed some features different from those of BSE in cattle (*B. taurus*).

Spongiform encephalopathies (SEs) are transmissible neurodegenerative diseases characterized by spongiform lesions and deposition of partially proteinase K–resistant prion protein (PrP^{sc}), a misfolded isoform of the normal host-encoded cellular prion protein (PrP^{c}), in the central nervous system (CNS). The oldest known SE is scrapie, which occurs naturally in sheep and goats. Since the onset of the bovine spongiform encephalopathy (BSE) epidemic in British cattle *(Bos taurus)* in 1986, novel SEs emerged in other animal species including domestic cats (*1*), a goat (*2*), primates (*3*), and several members of the families *Bovidae* and *Felidae* in zoos (*4*,*5*). Experimental and epidemiologic evidence indicate that these animals were infected by ingesting BSE-infected carcasses or meat and bone meal.

Zebus (*B. indicus*) belong to the family *Bovidae*. In Asia they are raised mainly as productive livestock, but in Europe they live primarily in zoos. We describe clinical, pathologic, and molecular features of the first case of SE in a zebu and address the question whether this animal was infected with the BSE agent.

The Study

In 2004, a 19-year-old miniature zebu in a zoo in Basel, Switzerland, fell during mating, after which it had abnormal gait and posture. After 6 weeks it started to bump into obstacles and showed anxiety and loss of proprioceptive control. Because of its old age and the progressive course of the disease, the animal was euthanized, and multiple organs were examined postmortem (Table 1). Histopathologic examination showed severe spongiform changes and a moderate gliosis in the brainstem (Figure 1A, nucleus of the solitary tract), and many other CNS structures. Immunohistochemical analysis (*6*), which used the monoclonal antibodies (MAbs) F99/97.6.1 (VMRD, Pullman, WA, USA) and P4 (R-biopharm, Darmstadt, Germany), identified a marked deposition of PrP^{sc} in the neuropil (granular type) and the neurons (Figure 1B and 1C). The cerebral cortex contained a moderately increased number of Alzheimer type II cells. Numerous nonnervous tissues, including the lymphoreticular system (Figure 1H), were analyzed by immunohistochemical techniques for the presence of PrP^{sc}, but none was found. Taken together, these findings led to the diagnosis of a severe SE in combination with a mild metabolic encephalopathy.

To assess the possibility that this animal was infected with the BSE agent, we compared the distribution of the SE-related histopathologic lesions and the PrP^{sc} deposits in different brain structures of the zebu to those in the brain of a Swiss BSE-affected cow. In both animals, spongiform lesions were similarly distributed throughout the brain, but overall the lesions in the zebu were more severe than those in the cow (Table 2). The depositions of PrP^{sc} in these structures, as determined by immunohistochemical analysis with MAb F99/97.6.1 and different commercial BSE screening tests (Check Western, Prionics, Zurich, Switzerland; TeSeE, Bio-Rad, Marnes-la-Coquette, France), were well associated with the histopathologic lesions in both animals (data not shown). In comparative Western immunoblot (WB) analysis that used MAb 6H4 (Prionics), the zebu CNS samples (Figure 2, lanes 3 and 5) showed a characteristic 3-band pattern representing un-, mono- and diglycosylated moieties of the proteinase K–resistant PrP^{sc} fragment. In the zebu these 3 bands clearly showed a migration pattern at a higher molecular mass than that of BSE in the cow (Figure 2, lanes 4 and 6) but similar to a sample from a sheep with scrapie (Figure 2, lane 7). When samples of the same animals were analyzed by WB (Figure 2) and immunohistochemical analysis (Figure 1C–E) with P4, an MAb used to discriminate between BSE and scrapie in sheep (*7*), PrP^{sc} was detectable in samples from the sheep with scrapie and the zebu under investigation but not in the cow with BSE. Sequencing of the open reading frame of the *Prnp* gene of the zebu confirmed that the encoded PrP protein was identical to the *B. taurus* PrP amino acid sequence (as translated from GenBank accession no. AJ298878).

Conclusions

In 1990, the first case of BSE in cattle in Switzerland was diagnosed; since then, authorities have banned meat and bone meal in ruminant feed in Switzerland. The zebu

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Table 1. Pathologic findings in a zebu with spongiform encephalopathy, Switzerland, 2004

Site	Finding
Vertebral column	Severe degeneration of intervertebral discs with ankylosing spondylarthrosis
Joints of extremities	Degenerative joint disease
Liver	Biliary cysts (affecting 60% of the liver)
Kidney	Tubular cysts; mild interstitial nephritis with glomerulosclerosis and tubular atrophy
Urinary bladder	Multiple papillomas
Abdominal cavity	Multiple foci of fat necrosis
Cardiovascular system	Mild coronary arteriosclerosis; mild valvular endocardiosis (mitral valve)
Mediastinal lymph nodes	Focal metastatic neuroendocrine tumor (origin unknown)
Central nervous system	Spongiform encephalopathy; metabolic encephalopathy (hepatoencephalopathy)

was born in 1985 and until 1990 ate commercial pellets containing meat and bone meal. Consequently, it might have been exposed to the BSE agent at ≤5 years of age.

The clinical signs of the zebu were specific for an SE but could have been explained partially by other pathologic findings, e.g., the degenerative lesions of the spine and the metabolic encephalopathy (Table 1). However, prominent spongiform changes and marked depositions of PrP^{sc} in the brain confirmed the clinical suspicion of an SE. The distribution and type of the lesions (Table 2) and PrP^{sc} deposits in the brain of the zebu were very similar to those in the brain of the Swiss BSE-affected cow and to findings that have been described previously for BSE in cattle in Switzerland (*8,9*) and elsewhere (*10–12*).

In contrast, molecular analysis of PrP^{sc} clearly showed a difference between the zebu and the BSE cow regarding 1) the apparent molecular mass of the PK-resistant fragment of PrP^{sc} on WB analysis and 2) the immunoreactivity with MAb P4 on WB and immunohistochemical analyses. Both observations can be explained by extended proteinase K cleavage at the N terminus of PrP^{sc} in cattle compared with the zebu, resulting in removal of the P4 epitope (*7*). Recently, very similar molecular findings were reported from France (*13*) in 3 exceptionally old (8, 10, and 11 years) cattle. These animals had an atypical PrP^{sc} WB profile, different from that traditionally seen in cattle with BSE but indistinguishable from those in sheep with natural scrapie and cattle with experimental scrapie. This molecular phenotype was retained after transmission of the disease to C57BL/6 mice (*14*). The authors speculated that their findings may reflect either an infection with another type of infectious agent distinct from BSE, e.g., scrapie, or a sporadic form of SE in cattle. For the zebu, the latter hypothesis is supported by the observation that the molecular features of PrP^{sc} were similar to the ones observed in type 1 sporadic Creutzfeldt-Jakob disease (*15*), an SE in humans. On the other hand, consistent with the findings on WB, MAb P4 readily detected PrP^{sc} by immunohistochemical analyses of the CNS of the zebu and in sheep with scrapie but not in bovine BSE under the conditions used.

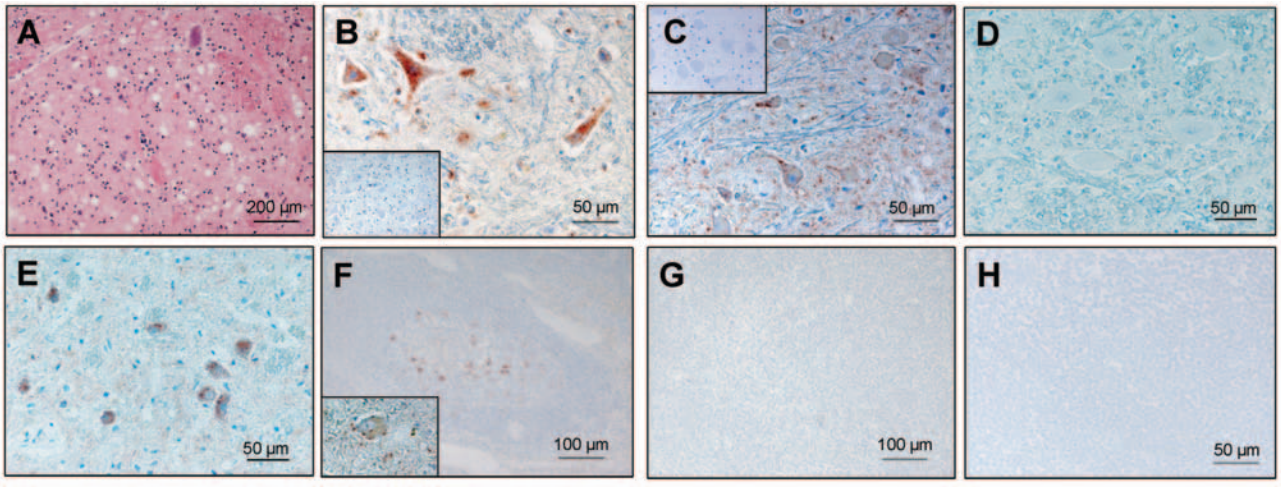

Figure 1. Histopathologic and immunohistochemical analyses. A) Spongiform lesions. B) Partially proteinase K–resistant prion protein (PrP^{SC}) deposits detected by immunohistochemistry (monoclonal antibody [MAb] F99/97.6.1 diluted 1:500) in the nucleus of the solitary tract (STN) in the zebu under investigation. C–E) Comparative immunohistochemistry with MAb P4 (1:800) in the olivary nuclei of the zebu (C), a bovine spongiform encephalopathy (BSE)-positive cow (D), and a scrapie-positive sheep (E). Insets show control tissue slides of BSE-negative cattle. F–H) Immunohistochemistry for PrP^{SC} in lymphoid tissue of the zebu (H, mediastinal lymph node), and a BSE-negative cow (G, mandibular lymph node) with MAb L42 (R-biopharm, 1:800). A retropharyngeal lymph node of a scrapie-affected sheep (F) and a brainstem tissue slide of the zebu (F, inset) served as positive controls. Pretreatment of the tissue slides comprised a proteinase K–digestion step (5 g/mL, 15 min, 37°C).

Table 2. Histopathologic lesions in brain of zebu with spongiform encephalopathy and cow with bovine spongiform encephalopathy, Switzerland, 2004*

Site	Cow	Zebu
Brainstem		
Dorsal motor nucleus of vagus nerve	+	++
Nucleus of spinal tract of trigeminal nerve	++	+++
Nucleus of hypoglossal nerve	+	++
Reticular formation	++	++
Nucleus of solitary tract	++	+++
Vestibular nuclei	++	+ to ++
Olivary nuclei	++	++
Cerebellar cortex	–	+
Midbrain (*substantia grisea centralis*)	+ to ++	++ to +++
Thalamus	++	++ to +++
Hippocampus	n.d.	+
Basal nuclei (*pallidum*)	+	+
Cerebral cortex	+	++

*–, absent; +, mild; ++, moderate; +++ severe; n.d., not done.

Extracellular and intracellular PrP^{sc} was detected by MAb P4 in the zebu and the sheep with scrapie. By contrast, in BSE-affected sheep, PrP^{sc} was detected by MAb P4 in extracellular but not intracellular space (*16*). However, further investigations that use comparative pathology and biologic strain typing would be required to characterize the phenotype of SE in this zebu and the infectious agent in more detail.

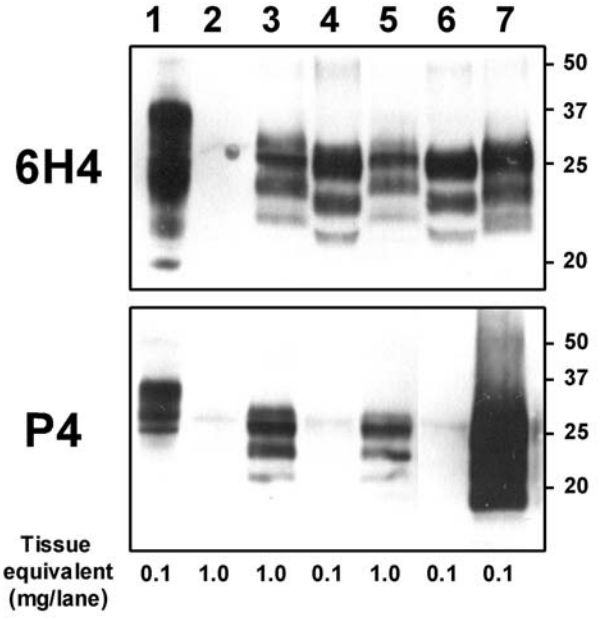

Figure 2. Molecular analyses of the zebu under investigation. Western immunoblot with monoclonal antibodies (MAbs) 6H4 (upper panel) and P4 (lower panel) after limited proteinase K digestion (100 µg/mL, 40 min, 48°C) of 10% brainstem (lanes 3 and 4) and thalamus (lanes 5, 6, and 7) tissue homogenates of the zebu (lanes 3 and 5), a cow with bovine spongiform encephalopathy (lanes 4 and 6), and a sheep with scrapie (lane 7). An undigested cattle brainstem tissue homogenate (lane 1) and a cerebrum tissue homogenate of a spongiform-encephalopathy-negative zebu (lane 2) were included as controls. All samples were processed equally as described by Stack et al. (7), and the membranes were exposed in parallel on the same photographic film. Molecular mass standards in kilodaltons are indicated on the right; tissue mass equivalents, at the bottom.

Whatever the origin of the disease, this case indicates that zebus are not naturally resistant to SE and, therefore, that *B. indicus* should be included in programs that monitor transmissible spongiform encephalopathies (TSEs) and in risk assessments in countries where these animals are part of the domestic livestock. Although the potential for this disease to cross the species barrier to other animals and humans is not known, zoos and veterinary services should be aware of the possibility of SEs in such animals so they can subsequently minimize the risk for foodborne SE infections in other animal species (especially *Felidae*) and humans by removing specified risk materials.

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Dr Seuberlich is a veterinarian and head of the TSE rapid test division in the Swiss reference laboratory for TSE in animals. His major research interests include the diagnosis and molecular characterization of emerging TSE agents.

References

1. Wyatt JM, Pearson GR, Smerdon TN, Gruffydd-Jones TJ, Wells GA. Spongiform encephalopathy in a cat [letter]. Vet Rec. 1990;125:513.
2. Eliot M, Adjou KT, Coulpier M, Fontaine JJ, Hamel R, Lilin T, et al. BSE agent signatures in a goat [letter]. Vet Rec. 2005;156:523–4. Erratum in Vet Rec. 2005;156:620.
3. Bons N, Mestre-Frances N, Belli P, Cathala F, Gajdusek DC, Brown P. Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents. Proc Natl Acad Sci U S A. 1999;96:4046–51.
4. Heim D, Geiser F, Perler L, Wyss R. Beyond BSE: Transmissible spongiform encephalopathies in other animal species. [Article in German]. Schweiz Arch Tierheilkd. 2002;144:664–73.
5. Kirkwood JK, Cunningham AA. Epidemiological observations on spongiform encephalopathies in captive wild animals in the British Isles. Vet Rec. 1994;135:296–303.
6. Organisation Mondiale de la Santé Animale. Manual of diagnostic tests and vaccines for terrestrial animals. 5th ed. 2004. [cited 2006 Oct 11].. Available from http://www.oie.int.
7. Stack MJ, Chaplin MJ, Clark J. Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies. Acta Neuropathol (Berl). 2002;104:279–86.
8. Fatzer R, Graber HU, Meyer RK, Cardozo C, Vandevelde M, Zurbriggen A. Neuronal degeneration in brain stem nuclei in bovine spongiform encephalopathy. Zentralbl Veterinarmed A. 1996;43:23–9.

9. Graber HU, Meyer RK, Fatzer R, Vandevelde M, Zurbriggen A. In situ hybridization and immunohistochemistry for prion protein (PrP) in bovine spongiform encephalopathy (BSE). Zentralbl Veterinarmed A. 1995;42:453–9.
10. Casalone C, Caramelli M, Crescio MI, Spencer YI, Simmons MM. BSE immunohistochemical patterns in the brainstem: a comparison between UK and Italian cases. Acta Neuropathol (Berl). 2006;111:444–9.
11. Simmons MM, Harris P, Jeffrey M, Meek SC, Blamire IW, Wells GA. BSE in Great Britain: consistency of the neurohistopathological findings in two random annual samples of clinically suspect cases. Vet Rec. 1996;138:175–7.
12. Wells GA, Wilesmith JW. The neuropathology and epidemiology of bovine spongiform encephalopathy. Brain Pathol. 1995;5:91–103.
13. Biacabe AG, Laplanche JL, Ryder S, Baron T. Distinct molecular phenotypes in bovine prion diseases. EMBO Rep. 2004;5:110–5.
14. Baron TG, Biacabe AG, Bencsik A, Langeveld JP. Transmission of new bovine prion to mice. Emerg Infect Dis. 2006;12:1125–8.
15. Parchi P, Castellani R, Capellari S, Ghetti B, Young K, Chen SG, et al. Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. Ann Neurol. 1996;39:767–78.
16. Jeffrey M, Martin S, Gonzalez L, Ryder SJ, Bellworthy SJ, Jackman R. Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE) and scrapie agents in sheep. J Comp Pathol. 2001;125:271–84.

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Drug Trafficking Routes and Hepatitis B in Injection Drug Users, Manipur, India

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Prevalence of hepatitis B genotype C in injection drug users in the northeastern Indian state of Manipur, neighboring the "Golden Triangle," correlates well with overland drug-trafficking routes, the injection drug use epidemic, and the spread of HIV. Further spread to other regions of India through mobile populations is possible.

Injection drug use is common in countries neighboring the "Golden Triangle" (Myanmar, Laos, and Thailand), known for heroin export to other countries. HIV and injection drug use outbreaks in countries neighboring the Golden Triangle, including the northeastern Indian state of Manipur, have been associated with drug-trafficking routes (*1*). Manipur shares a 358-km porous border with Myanmar. According to the National AIDS Control Organization, India (www.nacoonline.org), HIV infection rates among injection drug users in Manipur increased from 2%–3% in 1989 to >50% in 1991 and ≈64% in 2000. In addition, exposure rates of 100% for hepatitis B virus (HBV) and 92% for hepatitis C virus (HCV) have been detected among injection drug users in Manipur, and 95% of wives of injection drug users had HBV exposure (*2*). However, among both the users and their wives, prevalence of HBV genotypes and occult HBV infection (*3*), a known risk factor for hepatocellular carcinoma (HCC) (*4*), remained unknown.

HBV is classified into 8 genotypes, HBV/A through HBV/H, and is further divided into subgenotypes (*5*) that have a distinct geographic distribution and are associated with different disease outcomes. The geographic distribution of HBV is known to correlate with the anthropologic history of migration (*5*) and to the origin and routes of spread of HBV infection. In addition, behavioral patterns are known to change HBV genotype distribution in a region (*6*).

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Manipur is an important location, where mainland India (prevalent genotypes HBV/A, HBV/D) geographically meets China and Southeast Asia (prevalent genotypes HBV/B, HBV/C). Our study was designed to detect HBV DNA among injection drug users in Manipur and to analyze HBV genotypes for correlation with injection drug use and the HIV epidemic.

The Study

We examined HIV-positive injection drug users from Manipur who had been identified as anti-HBc–positive during previous serosurveys conducted by the National Institute of Cholera and Enteric Diseases (*2*). Serum samples (stored at –80ºC) taken from 63 men 18–25 years of age were available for the study. HBsAg detection was repeated with a monoclonal antibody–based Hepanostika hepatitis B surface antigen (HBsAg) kit (bioMérieux, Marcy l'Etoile, France). Anti-HCV antibody was detected using the Ortho HCV 3.0 test (Ortho-Clinical Diagnostics, Raritan, NJ, USA). We completed HBV DNA isolation, PCR amplification, genotype/subgenotype/subtype identification, recombination detection, and HBV DNA quantification with methods described earlier (*7*–*9*). We also compared nucleotide (online Appendix Table, available at http://www.cdc.gov/ncidod/EID/vol12no12/06-0425appT.htm) and deduced amino acid sequences (Table) with consensus sequence of amino acids of corresponding genotypes to detect substitutions (GenBank accession nos. DQ356432–DQ356441).

All those tested were HBsAg negative, and only 10 (15.9%) had detectable HBV DNA. Anti-HCV was detected in all but 1 sample (no. 4). Serum HBV DNA level was detectable in 5 of 10 samples (Table); the rest were below the detection limit of our assay.

HBV/C (all subtype adr except 1 adw2) was the predominant genotype, with 4 subgenotype C1 (HBV/C1) and 3 subgenotype C2 (HBV/C2) isolates. HBV/A1 adw2 was found in 1 of the isolates. Two other isolates (nos. 1 and 3, subtype adw2) indicated a possibility of intergenotypic recombination (Figure 1), but subgenotype could not be assigned for them.

BLASTN search (www.incogen.com/public_documents/vibe/details/NcbiBlastn.html) of sequence from sample no. 1 showed similarity to HBV/D as well as to HBV/A sequences from European countries. On the other hand, sequences from sample no. 3 showed similarity to HBV/C and HBV/A sequences from Southeast Asian countries and India. Simplot analysis confirmed recombination in these 2 isolates (Figure 2). Apart from the genotype-specific substitutions, deduced amino acid sequences did not have any remarkable escape mutant other than G145R in 2 isolates (Table).

Table. Serum HBV DNA levels and amino acid variability in the partial *s* gene, including the region encoding the major hydrophillic loop, compared with consensus sequences*

Isolate	Serum HBV DNA ($\times 10^3$ copies/mL)	110†	113†	114†	122‡	126†	131†	134†	143†	145	159†	160‡	161†	163
Cn A	–	I	S	T	K	T	N	F	T	G	A	K	Y	W
Cn D	–	–	–	S	–	–	T	Y	S	–	G	–	F	–
Cn C	–	L	T	S	–	I	T	–	S	–	–	R	F	–
IDU1	ND	–	–	–	–	–	–	–	S	–	G	–	F	–
IDU2	ND	–	–	–	–	–	–	–	–	–	–	–	F	–
IDU3	45.0	–	–	–	–	–	T	–	S	–	–	–	F	–
IDU4	14.0	L	–	S	–	I	T	–	S	–	–	R	F	–
IDU5	1.05	L	T	S	–	I	–	–	S	–	–	–	F	–
IDU6	ND	L	T	S	–	I	T	–	S	R	–	R	F	R
IDU7	ND	L	T	S	–	I	T	–	S	R	T	R	F	R
IDU8	9.0	L	T	S	–	I	T	–	S	–	–	R	F	–
IDU9	ND	L	T	S	–	I	T	–	S	–	–	R	F	–
IDU10	64.0	L	T	S	–	I	T	–	S	–	–	R	F	–

*Indicated by Cn, followed by genotype. HBV, hepatitis B virus; IDU, injection drug user; ND, not detectable by the assay.
†Genotype-specific sites.
‡Subtype-specific sites.

Conclusions

The data from this study showed occult HBV infection in 15.9% of the injection drug users tested. The rate of HBV DNA detection (10%–45%) was considerably different in studies reported from different cohorts of injection drug users in different countries, a finding that has been attributed to coinfection with HCV or low HBV DNA levels (*3*). Apart from HIV, our study group had a high frequency of HCV infection and low HBV DNA levels. Undetectable HBsAg, except in 2 cases with G145R substitution, may also be a result of the above-mentioned factors.

Although the importance of occult HBV infection is not well understood, a recent study reported occult HBV to be a significant risk factor for HCC, especially among persons who were anti-HBc–positive (*4*). Another recent study among HIV-infected patients documented death due to liver disease in 22% who were HBV coinfected, 44% who were HCV/HBV coinfected, and 15% who were HBV coinfected and had HCC (*10*). Thus, the clinical relevance in our study group also needs to be followed.

Although we detected a 100% prevalence of anti-HBc in our serosurveys (*2*), only 1 was HBsAg-positive. Therefore, the distribution of genotypes among those who were HBsAg positive could not be determined.

Findings of HBV/C1 (prevalent in China) appear to support the history of human migration from China to northeastern India. However, we did not detect HBV/B, also prevalent in China. Further, we did detect HBV/C2, which has close similarity to strains from Southeast Asian countries. The presence of HBV/C correlated well with drug-trafficking routes and the injection drug use epidemic. The geographic proximity of Manipur to the Golden Triangle, needle sharing among injection drug users, and drug traders thus contributed to the spread of HBV through drug-trafficking routes, similar to HIV (*1*).

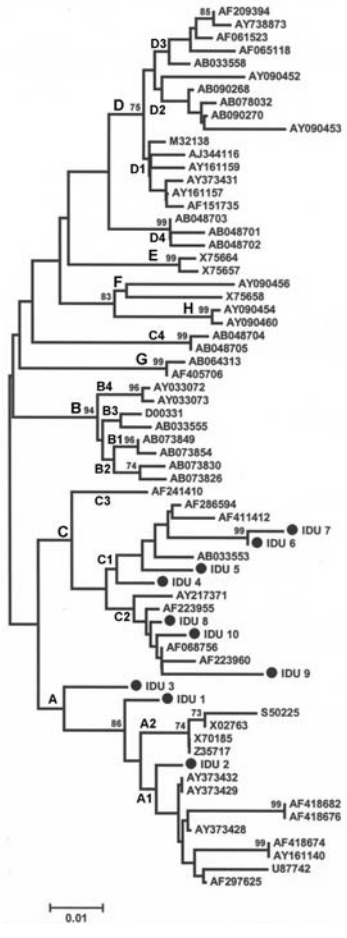

Figure 1. Phylogenetic relationships among the sequences of s gene from hepatitis B virus strains isolated in this study (shown with prefix "IDU") compared with reference sequences from GenBank (accession nos. are shown). Genotype and subgenotypes are indicated at each main branch and subbranch, respectively. Percentage of bootstrap replications supporting the clusters (≥75%) are also shown at the nodes.

In Manipur, HIV subtypes C and Thai B are prevalent (*11*); these are also prevalent in the India-Myanmar and China-Myanmar border regions. The presence of similar HIV subtypes among injection drug users in Manipur supports the presence of similar HBV strains (e.g., HBV/C1, HBV/C2) and their cotransmission through drug-trafficking routes. In addition, circulation of recombinant HBV is common among injection drug users because of repeated exposure (*12*). The intergenotypic HBV recombinants found in this study are thus expected. HBV/C has been associated with advanced liver disease and poses a higher risk for HCC in Asians (*13*) than does HBV/B. However, the clinical relevance of HBV recombinants and their pathogenesis is not well understood and needs further investigation.

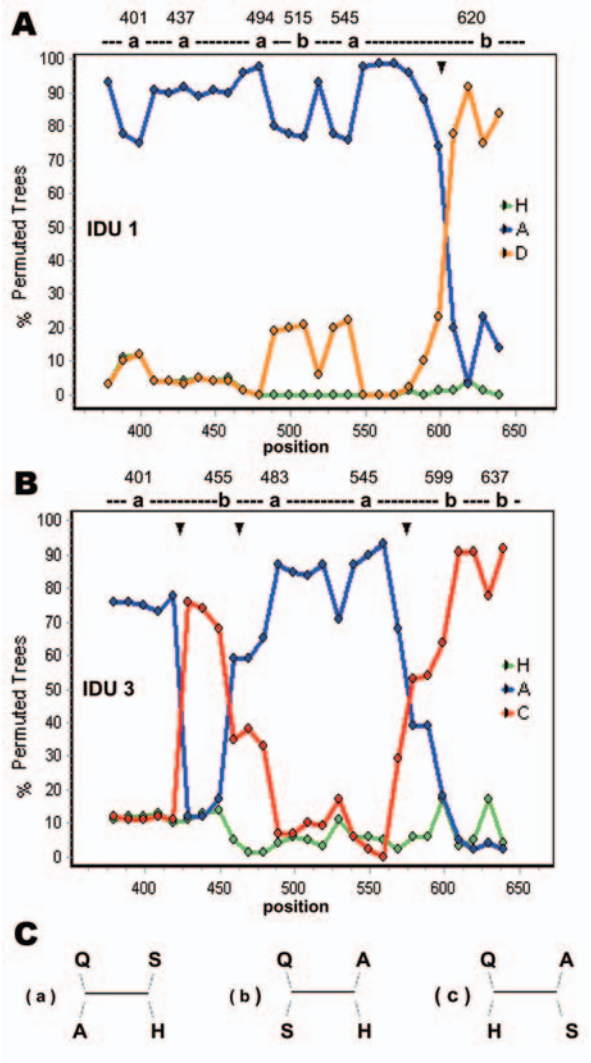

Figure 2. The location of recombination events in isolates IDU1 (A) and IDU3 (B), determined by using the bootscanning program of Simplot. Possible tree topologies (a,b, and c) are shown (C). The phylogenetically informative sites and the tree topologies supported at each of those sites are indicated over each plot. In the tree topologies, Q, A, and H indicate query sequences (IDU1 or IDU3), genotype A, and genotype H (outgroup) consensus sequences, respectively. S indicates consensus sequence of genotype D and genotype C for IDU1 and IDU3, respectively. Possible crossover points are indicated by solid triangles. A sliding window size of 60 bp, step size of 10 bp, Kimura-2 parameter, 1,000 bootstrap replicates, and neighbor-joining method were used for the analysis of recombination.

Poor, unemployed youths in the northeastern states of India are being recruited for drug trafficking to other regions (www.ipcs.org). Furthermore, national highways are associated with the prevalence of injection drug use in rural Manipur (*14*). As these highways connect Manipur with other parts of India, HBV/C may spread from Manipur to other parts of the country through persons who travel regularly, such as truck drivers and drug traffickers.

Presence of HBV genotypes A and D among patients from northern and western India is well documented. Recent research reported HBV/C with close similarity to Southeast Asian strains only from eastern India (*9*,*15*) and suggested injection drug users as a possible route of introduction (*15*). In addition, persons from northeastern India frequent Kolkata for education, employment, medical treatment, and other purposes. Studies on these mobile populations might provide further important information on the route, population at risk for infection, and changing epidemiology of these viral infections in other regions.

In conclusion, HBV/C, associated with severe liver disease in Southeastern Asia, may be emerging in the Manipur state of India through the trafficking routes of injection drugs. This genotype could spread to the general population through different modes. In light of growing information on the severity of liver disease in HBV-infected HIV/HCV patients, injection drug users should be the focus of additional education and healthcare efforts. The possibility of further spread of HIV/HBV/HCV through mobile populations to other regions of India warrants attention and further investigation.

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References

1. Beyrer C, Razak MH, Lisam K, Chen J, Lui W, Yu XF. Overland heroin trafficking routes and HIV-1 spread in south and south-east Asia. AIDS. 2000;14:75–83.
2. Saha MK, Chakrabarti S, Panda S, Naik TN, Manna B, Chatterjee R, et al. Prevalence of HCV and HBV infection amongst HIV seropositive intravenous drug users and their non-injecting wives in Manipur, India. Indian J Med Res. 2000;111:37–9.
3. Torbenson M, Kannangai R, Astemborski J, Strathdee SA, Vlahov D, Thomas DL. High prevalence of occult hepatitis B in Baltimore injection drug users. Hepatology. 2004;39:51–7.
4. Pollicino T, Squadrito G, Cerenzia G, Cacciola I, Raffa G, Crax A, et al. Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. Gastroenterology. 2004;126:102–10.
5. Norder H, Courouce AM, Coursaget JME, Lee SD, Mushahwar IK, Robertson BH, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes and HBsAg subtypes. Intervirology. 2004;47:289–309.
6. Kidd-Ljunggren K, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. J Gen Virol. 2002;83:1267–80.
7. Datta S, Banerjee A, Chandra PK, Chowdhury A, Chakravarty R. Genotype, phylogenetic analysis, and transmission pattern of occult hepatitis B virus (HBV) infection in families of asymptomatic HBsAg carriers. J Med Virol. 2006;78:53–9.
8. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, et al. Full length human immunodefficiency virus type 1 genomes from subtype C infected seroconverters in India, with evidence of intersubtype recombination. J Virol. 1999;73:152–60.
9. Banerjee A, Kurbanov F, Datta S, Chandra PK, Tanaka Y, Mizokami M, et al. Phylogenetic relatedness and genetic diversity of hepatitis B virus isolates in Eastern India. J Med Virol. 2006;78:1164–74.
10. Salmon-Ceron D, Lewden C, Morlat P, Bevilacqua S, Jougla E, Bonnet F, et al. The Mortality 2000 Study Group. Liver disease as a major cause of death among HIV infected patients: role of hepatitis C and B viruses and alcohol. J Hepatol. 2005;42:799–805.
11. Mandal D, Jana S, Bhattacharya SK, Chakrabarti S. HIV type 1 subtypes circulating in eastern and northeastern regions of India. AIDS Res Hum Retroviruses. 2002;18:1219–27.
12. Chen BF, Kao JH, Liu CJ, Chen DS, Chen PJ. Genotypic dominance and novel recombinations in HBV genotype B and C coinfected intravenous drug users. J Med Virol. 2004;73:13–22.
13. Chan HLY, Hui AY, Wong ML, Tse AML, Hung LCT, Wong VWS, et al. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut. 2004;53:1494–8.
14. Sarkar K, Panda S, Das N, Sarkar S. Relationship of national highway with injecting drug abuse and HIV in rural Manipur, India. Indian J Public Health. 1997;41:49–51.
15. Vivekanandan P, Abraham P, Sridharan G, Chandy G, Daniel D, Raghuraman S, et al. Distribution of hepatitis B virus genotypes in blood donors and chronically infected patients in a tertiary care hospital in southern India. Clin Infect Dis. 2004;38:e81–6.

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West Nile Virus in Horses, sub-Saharan Africa

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To evaluate the presence and extension of West Nile virus where French soldiers are stationed in Africa, specific antibody prevalence was determined by using ELISA and Western blot. Among 245 horses living in close proximity to the soldiers, seroprevalence was particularly high in Chad (97%) and Senegal (92%).

West Nile virus (WNV), a mosquito-transmitted flavivirus, was first isolated in Africa, West Nile district of Uganda, in 1937 (*1*). It has been shown to infect humans and a wide spectrum of animal species, including birds and horses. WNV infection is often inapparent or mild in humans but may cause severe and even fatal encephalitis in horses (*2*). Since 1999, dissemination of the virus through North America has reinforced interest in WNV epidemiology and evolution. Before 1999, outbreaks have been reported in North Africa, Israel, Romania, Russia, and France, where the virus may have been imported by migratory birds (*3–5*). However, few data are available on the current circulation of WNV in sub-Saharan Africa because of lack of surveillance and diagnostic tools in those countries.

Assessing and preventing human and zoonotic infectious diseases in tropical areas, particularly Africa, are essential missions of the French Defense Medical Service. To evaluate the presence and extension of WNV in the sub-Saharan African areas where French soldiers are stationed, serologic surveillance of horses living in close proximity was initiated in 2002.

The Study

From December 2002 through August 2005, blood samples were collected from 245 horses in 13 riding stables located in Senegal (Dakar, n = 25), Côte d'Ivoire (Abidjan, n = 95), Chad (N'Djamena, n = 30), Democratic Republic of the Congo (Kinshasa, n = 20), Gabon (Libreville, Port Gentil, and Moanda, n = 64), and Djibouti (Djibouti, n = 11) (Figure 1). Some horses were sampled twice in Chad (n = 18) and in Côte d'Ivoire (n = 18) during a period of 11–13 months. Origin, travel history, and how long the tested horses lived in the studied areas were not well known, but the horses were generally born and bred in the countries from which they were sampled (some of them in neighboring countries such as Burkina Faso, Mali, Niger, and Ethiopia), and none had a history of WNV vaccination.

Blood was centrifuged within 24 hours after collection. Serum was separated, frozen at –20°C, and sent to the virology laboratory of the Institut de Médecine Tropicale du Service de Santé des Armées in Marseille, France. Each sample was systematically tested for WNV-specific immunoglobulin G (IgG) by using an ELISA made in house. Antigen was prepared from a crude supernatant of Vero cells collected after 4 days of infection with WNV reference strain Eg 101 (viral titer >10^7 ID/mL) and treated with 1% Triton 100 and β-propiolactone (1:1,000). IgG was detected by using commercial peroxidase anti-horse IgG and tetramethylbenzidine as the substrate and standard procedures of ELISA capture. Serum specimens were considered positive for IgG when the optical density (OD) in

Figure 1. West Nile virus (WNV) circulation in Africa (*3,6–10*). Map of Africa summarizes published data related to WNV isolations, outbreaks, and sporadic or serologic cases (including this study). It also indicates the main bird migration routes (source: Wetlands International, Wageningen, the Netherlands). Source: Food and Agricultural Organization of the United Nations.

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antigen-positive wells was >0.3 and the ratio between the OD in corresponding antigen-positive wells and the mean OD in antigen-negative wells was >3.5. Because of the antigenic cross-reactivity among viruses of the *Flavivirus* genus, validation of ELISA IgG–positive samples was necessary. The plaque reduction neutralization test (PRNT) is the serologic reference method. All the IgG-positive sera collected during 2002–2003 were tested as described (*11*). In a 96-well plate, 4 dilutions of each serum sample (1:10, 1:40, 1:160, 1:640; 4 wells for each dilution) were incubated at 37°C for 1 hour in a viral suspension of 10–50 PFU in 50 µL before the addition of 100 µL of a Vero cell suspension (4×10^4/well). Four days later, the cell layer was fixed in formol and stained with crystal violet. A test result was considered positive for a dilution if the plaque reduction was >90% compared with the negative control. Because this method is fastidious and slow, we have used an alternative Western blot (WB) approach as described in Figure 2 (*12*).

Complete (100%) correlation between WB and PRNT and high specificity of WB were observed for a panel of 79 serum samples. Thus, only WB was used for validation of ELISA IgG-positive sera for the 2004–2005 samples. All serum samples that were positive for WNV IgG were further investigated using immunocapture IgM ELISA to evaluate the time of infection.

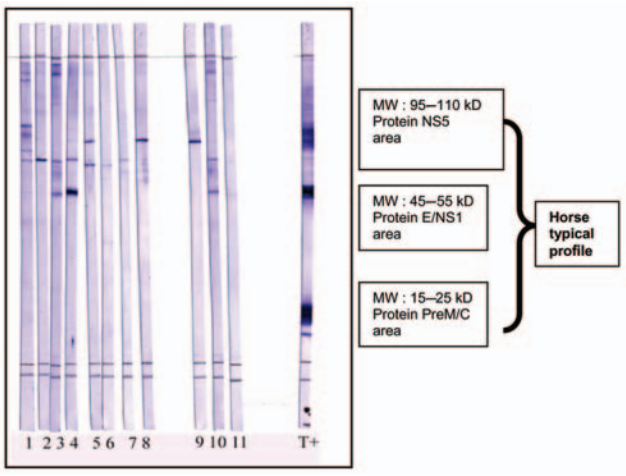

Figure 2. West Nile virus (WNV) Western blot as a validation of ELISA-positive results. Proteins from WNV-infected cells lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 13% acrylamide gels and transferred on polyvinylidene difluoride membranes. Strips were cut and blocked with nonfat milk (5%). Serum samples diluted (1:200) in phosphate-buffered saline (PBS), 5% nonfat milk, and 0.1% Tween 20 were loaded on strips and incubated for 1 h with slow shaking. Strips were washed 4 times in PBS, 0.5% Tween, and incubated for 1 h in anti-horse immunoglobulin G (IgG) peroxidase (1:8,000). After 4 washes, Western blots were incubated for 5 min with trimethylbenzidine substrate (with specific enhancers). Strips were washed once with water to stop the staining. Results were obtained within 24 h. MW, molecular weight; T+, horse from Chad with positive Western blot results; serum samples 1–11, horse from southeast France, IgG positive by ELISA but negative by neutralization; NS5, nonstructural protein 5; E, envelope; PreM, premembrane; C, capsid.

Conclusions

Except in Gabon (3%), high seroprevalence (28%–97%) for WNV was detected in horses in West Africa and Central Africa, especially in N'Djamena (97%) and Dakar (92%) (Table 1). Seroprevalence of 9% was detected in East Africa (Djibouti).

All horses positive for IgG were negative for IgM, which indicates relatively old infection. Estimating the date of onset of WNV infection is difficult because of a lack of published data relative to WNV IgM and IgG response in naturally infected horses; only persistence of IgG several years after infection has been described (*4*). Because histories of tested horses are not well known, determining precisely when and where horses became infected is difficult. However, infections likely occurred in sampling countries or neighboring sub-Saharan African countries.

Seroconversion from negative to positive was found in 2 horses in Chad (Table 2) from 2003 through 2004, while 5 of 15 seropositive horses became seronegative, which suggests maintenance of an enzootic cycle in this area but

Table 1. West Nile virus antibody prevalence in horses in 6 African countries, December 2002–August 2005*

Country (sampling sites)	Sampling date (no. riding stables)	No. tested	No. IgG+†	No. confirmed IgG+‡	% Seroprevalence
Senegal (Dakar)	Dec 2002, Apr 2003 (1)	25	23	23	92
Côte d'Ivoire (Abidjan)	Dec 2003, Dec 2004, Jan 2005 (3)	95	51	27	28
Chad (N'Djamena)	Nov 2003, Oct 2004 (2)	30	29	29	97
DRC (Kinshasa)	Jul 2004 (1)	20	9	6	30
Gabon (Libreville, Port Gentil, Moanda)	Dec 2004 (4)	64	9	2	3
Djibouti (Djibouti)	Jul 2004, Aug 2005 (2)	11	2	1	9
Total		245	123	88	36

*IgG+, positive for immunoglobulin G; DRC, Democratic Republic of the Congo.
†By ELISA.
‡By Western blot and seroneutralization for samples from 2002–2003, by Western blot only for samples from 2004 to 2005.

Table 2. Results of follow-up testing for West Nile virus in horses in Chad and Côte d'Ivoire

	Initial testing*	Follow-up testing†
Chad (n = 18)	3 negative	1 negative, 2 positive
	15 positive	5 negative, 10 positive
Côte d'Ivoire (n = 18)	8 negative	8 negative
	10 positive	9 negative, 1 positive

*November 2003 in Chad and December 2003 in Côte d'Ivoire. Positive results were confirmed by Western blot and seroneutralization.
†October 2004 in Chad and December 2004–January 2005 in Côte d'Ivoire. Positive results were confirmed by Western blot.

at a low level. During the same period in Côte d'Ivoire, 9 of 10 previously seropositive horses were seronegative, while none of seronegative horses became seropositive. The most probable explanation is a decrease in IgG titer under the retained threshold of positivity compatible with the decrease of WNV IgG response in horses, which suggests the presence of an older epizootic in this area.

The immunoblotting method is a fast and specific confirmation assay for validation of ELISA WNV IgG–positive sera. Once validated by further studies, WB could be used as an alternative to PRNT.

Serologic data from our study should be considered as evidence of WNV activity in sub-Saharan Africa, which has a potential risk for populations and foreigners, including French soldiers. Previously, WNV was known to circulate in mosquitoes and some bird species without having any clear pathogenicity; outbreaks have been reported only in South Africa and in the Democratic Republic of the Congo (*5*,*6*). Before our study, no data relative to WNV circulation in horses in sub-Saharan Africa were documented, and WNV activity had never been reported in Chad or Gabon.

Highest (92%–97%) seroprevalence was found in the western and central parts of the Sahelian area (Dakar and N'Djamena). This area, characterized by a semiarid climate and vegetation of steppe and brush grass, is the most frequently involved area for WNV isolations in birds and mosquitoes (*7*,*13*). The seroprevalence was lower in the east of the Sahelian area (Djibouti, 9%), where the climate is arid and the vegetation is semidesert, and in the sub-Sahelian area (3%–30%), where the vegetation is tropical rain forest or woodland savanna in a humid or semihumid climate. That forest favors the sedentariness of birds has been documented (*14*). The migration of birds may certainly be enhanced in the Sahelian area; the introduction of WNV by migratory birds during their flight between Senegal and Europe has been suspected as a cause of the 1996 outbreak in Morocco (*10*). To estimate possibilities of incursions of WNV, especially in Eurasia, effects of environmental factors such as climate and vegetation on reservoir and vector populations in sub-Saharan Africa should be precisely studied.

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Dr Cabre is a veterinarian in the French Defense Medical Service. He was on duty in Chad during the end of 2003, where he developed an interest in known and emerging infectious diseases, especially zoonoses, that occur in areas of French military operations.

References

1. Smithburn KC, Hughes TP, Burke AW, Paul JH. A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med Hyg. 1940;20:471–92.
2. Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ. West Nile virus. Lancet Infect Dis. 2002;2:519–29.
3. Hubalek Z, Halouzka J. West Nile fever: a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis. 1999;5:643–50.
4. Murgue B, Murri S, Zientara S, Durand B, Durand JP, Zeller HG. West Nile outbreak in horses in southern France, 2000: the return after 35 years later. Emerg Infect Dis. 2001;7:692–6.
5. Zeller HG, Schuffenecker I. West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas. Eur J Clin Microbiol Infect Dis. 2004;23:147–56.
6. Nur YA, Groen J, Heuvelmans H, Tuynman W, Copra C, Osterhaus AD. An outbreak of West Nile fever among migrants in Kisangani, Democratic Republic of Congo. Am J Trop Med Hyg. 1999;61:885–8.
7. Centre Collaborateur OMS de Référence et de Recherche sur les Arbovirus. Flavivirus, West Nile: 282 souches identifiées. 1962 [updated 2005 Jul; cited 2006 Aug 17]. Available from http://www.pasteur.fr/recherche/banques/CRORA/virus/v010100.htm
8. Murgue B, Murri S, Triki H, Deubel V, Zeller HG. West Nile in the Mediterranean basin: 1950–2000. Ann N Y Acad Sci. 2001;951:117–26.
9. Murgue B, Zeller H, Deubel V. The ecology and epidemiology of West Nile virus in Africa, Europe and Asia. Curr Top Microbiol Immunol. 2002;267:195–221.
10. Schuffenecker I, Peyrefitte CN, El Harrak M, Murri S, Leblond A, Zeller HG. West Nile virus in Morocco, 2003. Emerg Infect Dis. 2005;11:306–9.
11. De Madrid AT, Porterfield JS. A simple micro-culture method for the study of group B arboviruses. Bull World Health Organ 1969;40:113–21.
12. Prangé A, Coussinier-Paris P, Davoust B, Cabre O, Gravier P, Sanson Y, et al. Diagnostic différentiel de l'infection par le virus West Nile: intérêt de l'immunoblot. Proceedings of the 11th Actualités du Pharo Conference; 2004 Sep 9–11; Marseilles, France. Med Trop (Mars). 2004;64:293.
13. Traore-Lamizana M, Zeller HG, Mondo M. Isolations of West Nile and Bagaza viruses from mosquitoes (Diptera: *Culicidae*) in center Senegal (Ferlo). J Med Entomol. 1994;31:934–8.
14. Brown LH, Urban EK, Newman K, editors. The birds of Africa. Vol.1. 1st ed. New York: Academic Press; 1982.

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Nonpharmaceutical Influenza Mitigation Strategies, US Communities, 1918–1920 Pandemic

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We studied nonpharmaceutical interventions used to mitigate the second, and most deadly, wave of the 1918–1920 influenza pandemic in the United States. We conclude that several small communities implemented potentially successful attempts at preventing the introduction of influenza.

The 1918–1920 influenza pandemic was the deadliest pandemic in human history (*1*–*6*). We undertook a historical evaluation of nonpharmaceutical interventions (NPIs) during that pandemic (*7*), with an emphasis on American communities during the second wave (September–December 1918). The full report and a digital archive of primary sources for this study can be accessed online (available from http://www.med.umich.edu/medschool/chm/influenza).

The Study

We selected 6 US communities that reported relatively few, if any, cases of influenza and no more than 1 influenza-related death while NPIs were enforced during the second wave of the 1918 pandemic: San Francisco Naval Training Station, Yerba Buena Island, California; Gunnison, Colorado; Princeton University, Princeton, New Jersey; Western Pennsylvania Institution for the Blind, Pittsburgh, Pennsylvania; Trudeau Tuberculosis Sanatorium, Saranac Lake, New York; and Fletcher, Vermont (Table). We also studied the college community of Bryn Mawr College, Bryn Mawr, Pennsylvania, which took several intensive NPI measures and experienced no deaths during the second wave but did encounter a high case rate (25% of its student body). We identified these sites first by consulting Jordan's 1927 text, Epidemic Influenza (*1*). We then verified and modified this list by reviewing 240 federal, 92 state (from 40 states), and 25 special local reports and documents. We conducted in situ archival research at 34 locations and examined >1,400 newspaper and contemporary medical and scientific journal articles for the 1918–1920 period.

The communities we identified were diverse and had unique characteristics. Fletcher, Vermont (population 737), was simply too small to suggest that its success resulted from anything more than remote location, good fortune, or the ways in which the virus skipped some communities altogether for unknown reasons (*8*–*10*). The Trudeau Tuberculosis Sanitarium (*9*) and the Western Pennsylvania Institution for the Blind (*10*) were already de facto quarantine islands because of the era's prevailing views toward confinement of the contagious and the disabled. Princeton University provided a good example of how a social institution with some measure of control over its population might implement NPIs to protect itself (*11*).

The US Naval Base at Yerba Buena Island in San Francisco Bay (*12*) and the mining town of Gunnison, Colorado (*13*), also offer potential lessons for contemporary pandemic influenza preparedness planning. Under the direction of public health officers, the still-healthy island and mountain town essentially cut off all contact with the outside world to shield themselves from the incursion of influenza. The 2 sites saw almost no cases of infection and thus experienced no deaths, for 2 and 4 months, respectively.

Most important, these communities enacted a policy we have termed protective sequestration, or the measures taken by the authorities to protect a defined and still-healthy population from infection before it reaches that population. These measures include the following: 1) prohibitions on members of the community from leaving the site; 2) prohibitions against visitors from entering a circumscribed perimeter; 3) typically placing in quarantine those visitors who are allowed to enter for a period of time before admission; and 4) if available, taking advantage of geographic barriers, such as an island or remote location.

Several themes emerged from our historical research. First, coordination among public agencies is essential to any effective public health response. Despite some tension among city, county, and state officials in Gunnison, their relatively smooth cooperation may have played a role in their implementing and maintaining strict public health measures. Second, neither Gunnison nor Yerba Buena could have escaped the flu without full cooperation from the local population. Gunnison's low population density and self-sufficient ranching lifestyle made it easier for residents to bide their time (Figure 1). At Yerba Buena, the military chain of command mandated the cooperation of

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Table. Six communities that along with Bryn Mawr College escaped influenza pandemic, 1918–1920

Characteristics	Yerba Buena, CA	Gunnison, CO	Princeton University, NJ	WPIB, PA*	Trudeau Sanatorium, NY	Fletcher, VT	Bryn Mawr College, PA
Population	≈ 6,000	1,329 in town, 5,590 in county	1,142	179 students; faculty and staff also lived on-site	356 patients admitted in 1918; 259 discharged; average daily patient census of 150	737	465
Geographic isolation	Small island off coast of San Francisco	Small mountain community in western Colorado; commercial, educational, and transportation hub	Student body in a small college town; campus somewhat separated from the town	Located in a busy residential Pittsburgh neighborhood but somewhat isolated by standards of the day	A small institute on the outskirts of a very small mountain community in upstate New York	Very small rural community in upstate Vermont	Student body in a small college town; only 10 miles from Philadelphia
Ordinary or special population	Primarily a military population; ≈1,000 civilian family members and workers were present	Ordinary population composed of native-born and immigrant residents	All-male student body; 92% of students were members of a military training corps	Student body was blind and thus isolated by standards of the day	Patients and staff were tubercular and were thus isolated by standards of the day	Ordinary rural population	All-female student body
Period of protective sequestration	Sep 23 – Nov 21, 1918	Oct 31, 1918 – Jan 20, 1919 (countywide); public closures and imposed social distancing as of Oct 8, 1918	Never under a full protective sequestration, as recruits and cadets continually arrived and left; restrictions on off-campus travel (with perimeter control) imposed Oct 8–Dec 21, 1918	Early Oct – late Nov 1918	A de facto protective sequestration existed due to its geographic and institutional isolation	Not applicable	≈Oct 1 – Nov 7, 1918
Cases and deaths	0 cases, 0 deaths during protective sequestration	0 cases, 0 deaths in town (2 cases, 1 death in county)	68 cases, 0 deaths in student population†	12 cases, 0 deaths	0 cases, 0 deaths	2 cases, 0 deaths	110 cases, 0 deaths

*WPIB, Western Pennsylvania Institution for the Blind.
†One professor died.

sailors and allowed the island's commander to close off the base from the outside world with little interference (Figure 2). Finally, these communities had the advantage of early warnings to prepare their populations. Both tracked influenza's westward movement from August to September and, unlike communities along the East Coast, could implement protective health strategies before cases appeared at their doorsteps.

One would like to think that the 6 communities we identified fared better than others because of the NPIs they enacted. While we cannot prove this for any of them, the case is perhaps strongest for Yerba Buena and possibly Gunnison. Further complicating our task, in addition to the uneven quality and quantity of information available for study, is that some of these communities were sparsely populated and geographically isolated, and all of them were subject to the vagaries of how the influenza virus affected populations. Indeed, these communities represent the exception rather than the rule in terms of how most American communities experienced the influenza pandemic of 1918–1920 (*14,15*). This leads to several intriguing questions regarding what these escape communities can teach us about pandemic preparations today, let alone the question of whether such measures can even be replicated.

Figure 1. Western State College, Gunnison, Colorado. Source: Denver Public Library, Western History Collection, call no. X-9302.

Conclusions

First, protective sequestration, if enacted early enough in the pandemic, crafted so as to encourage the compliance of the population involved, and continued for the lengthy time period in which the area is at risk, stands the best chance of guarding against infection. Second, available data from the second wave of the 1918–1920 influenza pandemic fail to show that any other NPI (apart from protective sequestration) was, or was not, effective in preventing the spread of the virus. Despite implementing several NPIs, most communities sustained considerable illness and death. We could not assess how the timing of NPI implementation across the nation affected disease mitigation efforts nor whether these NPIs lessened what might have been even higher rates had these measures not been in place in various locations. Moreover, we could not locate any consistent, reliable data supporting the conclusion that face masks, as available and as worn during the 1918–1920 influenza pandemic, conferred any protection to the populations that wore them (*16*). In fact, evidence suggests that in most American communities NPIs did not prevent the spread of virus in 1918. What remains unclear is the extent to which they may have been partially effective in reducing spread or mitigating community impact.

Figure 2. Naval Training Station, San Francisco, California. View looking south over the wharf area, from the eastern end of Yerba Buena ("Goat") Island, 1921. Long Wharf is in the foreground, lined with rowing boats on davits. Beyond is Navy Wharf, with the receiving ship *Boston* (1887-1946) at far left. The Lighthouse Wharf is beyond that. Collection of Eugene R. O'Brien. Photo #NH 100361. US Naval Historical Center photograph. From US Naval Historical Center (available from http://www.history.navy.mil/photos/images/i00000/i00361.jpg).

However inconclusive the data from 1918 are, the collective experiences of American communities from the pandemic are noteworthy, especially in light of the fact that, if faced with a pandemic today, we would likely rely on many of these same NPIs to attempt to mitigate the spread of the infection until pharmacological supplies of vaccine and antiviral agents were available (*17–19*). It is true that the United States of today is a much different nation than it was in 1918, with a larger, more mobile, and more complex society. It is equally true that the communities we examined were all small and relatively isolated (or isolatable). Nevertheless, in the event of another influenza pandemic, many specific subcommunities (e.g., military installations, college and university campuses, nursing homes) may wish to consider protective sequestration measures as potential means to prevent or delay the onset of epidemic influenza in their populations.

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References

1. Jordan EO. Epidemic influenza: a survey. Chicago: American Medical Association; 1927.
2. Crosby AW. America's forgotten pandemic: the influenza of 1918. New York: Cambridge University Press; 2003.
3. Byerly CR. Fever of war: the influenza epidemic in the U.S. Army during World War I. First ed. New York: New York University Press; 2005.
4. Hoehling AA. The great epidemic. Boston: Little, Brown and Company; 1961.
5. Kolata G. Flu: the story of the great influenza pandemic of 1918 and the search for the virus that caused it. New York: Farrar, Straus and Giroux; 1999.
6. Barry JM. The great influenza: the epic story of the deadliest plague in history. New York: Viking; 2004.
7. World Health Organization Writing Group. Nonpharmaceutical interventions for pandemic influenza, international measures. Emerg Infect Dis. 2006;12:81–94.
8. Vermont state board of health. Twenty-second [twelfth biennial] report of the state board of health of the state of Vermont from January 1, 1918, to December 31, 1919. Rutland (VT): The Turtle Company, Marble City Press Publishers; 1920.

9. Taylor R. Saranac: America's magic mountain. Boston: Houghton-Mifflin; 1986.
10. Johnston JI. History and epidemiology of influenza. In: University of Pittsburgh School of Medicine. Studies on epidemic influenza. Pittsburgh: University of Pittsburgh School of Medicine; 1919.
11. Taylor JM. An exploit in control of influenza. Boston Med Surg J. 1920;182:601.
12. Annual reports of the Navy Department for the fiscal year 1919. Washington: Government Printing Office, 1920; 2482–6.
13. Leonard SJ. The 1918 influenza epidemic in Denver and Colorado. Essays in Colorado History. 1989;9:9.
14. Sydenstricker E. Preliminary statistics of the influenza epidemic. Public Health Rep. 1918;33:2305–21.
15. Frost WH. The epidemiology of influenza. Public Health Rep. 1919;34:1823–36.
16. Kellogg WH. Influenza: a study of measures adopted for the control of the epidemic. California State Board of Health, Special bulletin no. 31. Sacramento (CA): California State Printing Office; 1919.
17. Ferguson NM, Cummings DA, Fraser C, Cajka CJ, Cooley PC, Burke DS. Strategies for mitigating an influenza pandemic. Nature. 2006;442:448–52.
18. Ferguson NM, Fraser C, Donnelly CA, Ghani AC, Anderson RM. Public health risk from the avian H5N1 influenza epidemic. Science. 2004;304:968–9.
19. Longini IM, Nizam A, Xu S, Ungchusak K, Hanshaoworakul W, Cummings DA, et al. Containing pandemic influenza at the source. Science. 2005;309:1083–7.

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Fatal Human Infection with Rabies-related Duvenhage Virus, South Africa

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Duvenhage virus was isolated from a patient who died of a rabieslike disease after being scratched by a bat early in 2006. This occurred ≈80 km from the site where the only other known human infection with the virus had occurred 36 years earlier.

The genus *Lyssavirus* within the family *Rhabdoviridae* currently includes rabies virus (RABV) (genotype 1) and 6 rabies-related viruses: 3 from Africa, Lagos bat virus (LBV) (genotype 2), Mokola virus (MOKV) (genotype 3), and Duvenhage virus (DUVV) (genotype 4); European bat lyssaviruses 1 and 2 (EBLV1 and 2) (genotypes 5 and 6); and Australian bat lyssavirus (ABLV) (genotype 7) (*1*). Strains of RABV (genotype 1) undergo genetic adaptation to particular animal hosts so that within specific areas the disease is manifested and transmitted predominantly by 1 host species. The canid, or dog, biotype of RABV is the most widely distributed in the world. In South Africa, RABV is transmitted by dogs and jackals in the northern region of the country, by dogs in the eastern region where most cases of human rabies occur, and by bat-eared foxes in the western region. In addition, an indigenous herpestid biotype of RABV (genotype 1) is transmitted by mongooses (*Herpestidae*) on the interior plateau of South Africa. This biotype does not spread readily to dogs but causes occasional cases of rabies in dogs, cats, humans, and more frequently, cattle and sheep (*2*).

RABV (genotype 1) has never been isolated from bats outside North and South America, but rabies-related viruses have been isolated from bats elsewhere. In Africa, LBV and DUVV are associated with bats, but MOKV is uniquely associated with shrews and rodents, not bats. Fifteen isolations of LBV have been reported, including 8 from fruit bats and a cat in KwaZulu-Natal Province of South Africa, but the virus has never been associated with human disease (*2*,*3*). MOKV has been isolated from shrews, rodents, cats, and a dog in Africa and from 7 cats with rabies-like disease in KwaZulu-Natal and Eastern Cape provinces of South Africa (*2*). The virus is believed to have caused rabieslike disease in 2 persons in Nigeria in 1969 and 1971, shortly after its initial discovery in shrews in 1968, but no cases of human infection have subsequently been recognized (*4*,*5*). DUVV was discovered in 1970 when it caused fatal rabieslike disease in a person bitten by an unidentified insectivorous bat ≈150 km northwest of Johannesburg, South Africa (*6*). In 1981, the virus was isolated from what is believed to have been a *Miniopterus schreibersi* insectivorous bat caught in daylight by a cat in Makhado town (formerly Louis Trichardt) in Limpopo Province, South Africa, and in 1986 the virus was recovered from an insectivorous bat, *Nycteris thebaica*, trapped in a survey in Zimbabwe (*7*,*8*).

The Study

DUVV infection was recently confirmed in a 77-year-old man with type 2 diabetes who was scratched on the face by what appears to have been an insectivorous bat in February 2006 in North West Province, South Africa, ≈80 km from the location where the first DUVV infection occurred 36 years earlier. The bat flew into a room at night, landed on the man's spectacles while he was attempting to chase it out, and scratched his face as he brushed it off. The bat did not appear to have bitten him, and it escaped after the incident. He did not seek medical care, and thus no postexposure treatment was given. He became ill at home in Cape Town 27 days later and received treatment for influenzalike illness. He slept most of the following day, had hallucinations that night, and was admitted to a hospital on the third day of illness. On admission, he had a fever (40°C), tachycardia, neck and general limb rigidity, hyperreflexia, facial fasciculation, and involuntary grimacing. Within 24 hours generalized tonic-clonic seizures had developed with status epilepticus supervening. These necessitated intubation, sedation, and mechanical ventilation. He died on day 14 of his illness.

Heminested reverse transcription–PCR was performed as previously described (*9*) with modified forward primer JW12 (*10*). This procedure detected lyssavirus nucleic acid in saliva taken on day 10 of illness and in brain tissue collected postmortem. Nucleotide sequencing of the PCR products and phylogenetic analysis performed as previously described (*11*) confirmed the identity of the agent as DUVV (Figure), and live virus was isolated from saliva and brain tissue by mouse inoculation. Immunofluorescence tests with antirabies conjugate prepared to be

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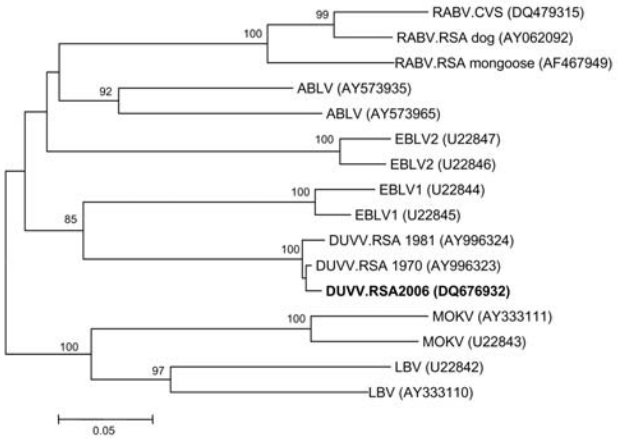

Figure. Neighbor-joining tree relating a 372-bp nucleotide sequence of the nucleoprotein gene of the recent Duvenhage virus (DUVV) isolate (**boldface**) to representative sequences of the known lyssavirus genotypes, including South African dog and mongoose isolates and the reference challenge virus strain (CVS) of rabies virus (RABV) (GenBank accession nos. are indicated in parentheses). Bootstrap values were determined by 1,000 replicates. ABLV, Australian bat lyssavirus; EBLV, European bat lyssavirus; MOKV, Mokola virus; LBV, Lagos bat virus.

cross-reactive with the African rabies–related viruses (Onderstepoort Veterinary Institute, Pretoria, South Africa) showed small and sparse inclusion bodies in impression smears of the cortex, hippocampus, thalamus, medulla, and cerebellum. Histopathologic examination of sections from the cortex, hippocampus, thalamus, hypothalamus, midbrain, pons, medulla, and cerebellum showed polioencephalitis affecting predominantly the diencephalon and brainstem and involving varying degrees of neuronopathy, neuronal loss, astrocytosis, parenchymal and perivascular lymphocytic infiltration with CD45 immunopositivity, sparse macrophage activation, and axonal spheroid formation. No nuclear or cytoplasmic inclusions were observed.

Conclusions

The ability to distinguish between various lyssaviruses and monitor their relative distribution and prevalence has important implications for implementation of control measures. It was recognized in 1932 that mongoose-associated rabies in South Africa differs from classic dog rabies (*2*). Although an inadequately characterized lyssavirus was isolated from a bat trapped in a survey in 1963, before the existence of rabies-related viruses was known, awareness of lyssaviruses other than rabies viruses dates from the identification of DUVV in 1970 and was followed by detection of LBV and MOKV in South Africa (*6*,*7*,*12*). Routine differentiation of diagnostic isolates became feasible with the availability of monoclonal antibodies during the 1980s and the subsequent introduction of molecular epidemiology techniques (*7*,*13*–*15*). Although instances of persons seeking rabies prophylaxis after exposure to bats have been reported, the recent case of DUVV infection constitutes only the second known instance of a person in South Africa with lyssavirus infection after such an encounter. Nevertheless, it is clear that rabies-related viruses are widely endemic in South Africa and that active investigation of the bat-associated lyssaviruses is warranted.

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References

1. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. Virus taxonomy: the classification and nomenclature of viruses. The eighth report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press; 2004. p. 623–31.
2. Swanepoel R. Rabies. In: Coetzer JAW, Tustin RC, editors. Infectious diseases of livestock. 2nd ed. Cape Town (South Africa): Oxford University Press; 2004. p. 1123–82.
3. Markotter W, Randles J, Rupprecht CE, Sabeta CT, Wandeler AI, Taylor PJ, et al. Recent Lagos bat virus isolations from bats (suborder Megachiroptera) in South Africa. Emerg Infect Dis. 2006;12:504–6.
4. Familusi JB, Moore DL. Isolation of a rabies related virus from the cerebrospinal fluid of a child with 'aseptic meningitis.' Afr J Med Sci. 1972;3:93–6.
5. Familusi JB, Osunkoya BO, Moore DL, Kemp GE, Fabiyi A. A fatal human infection with Mokola virus. Am J Trop Med Hyg. 1972;21:959–63.
6. Tignor GH, Murphy FA, Clark HF, Shope RE, Madore P, Bauer SP, et al. Duvenhage virus: morphological, biochemical, histopathological and antigenic relationships to the rabies serogroup. J Gen Virol. 1977;37:595–611.
7. Schneider LG, Barnard BJH, Schneider HP. Application of monoclonal antibodies for epidemiological investigations and oral vaccination studies: I. African virus. In: Kuwert E, Mérieux C, Koprowski H, Bögel K, editors. Rabies in the tropics. Berlin: Springer-Verlag; 1985. p. 47–59.
8. Foggin CM. Rabies and rabies-related viruses in Zimbabwe: historical, virological and ecological aspects. [Doctoral dissertation]. Harare (Zimbabwe): University of Zimbabwe; 1988.
9. Whitby JE, Heaton PR, Whitby HE, O'Sullivan E, Johnstone P. Rapid detection of rabies and rabies-related viruses by RT-PCR and enzyme-linked immunosorbent assay. J Virol Methods. 1997;69:63–72.
10. Black EM, McElhinney LM, Lowings JP, Smith J, Johnstone P, Heaton PR. Molecular methods to distinguish between classical and the rabies-related European bat lyssaviruses. J Virol Methods. 2000;87:123–31.
11. Venter M, Smit S, Leman P, Swanepoel R. Phylogenetic evidence of widespread distribution of genotype 3 JC virus in Africa and identification of a type 7 isolate in an AIDS patient. J Gen Virol. 2004;85:2215–9.
12. Mansvelt PR. The role of wild life in the epizootiology of some infectious and parasitic animal diseases in South Africa. Bull Off Int Epizoot. 1965;64:825–35.

13. Smith JS, Yager PA, Orciari LA. Rabies in wild and domestic carnivores of Africa: epidemiological and historical associations determined by limited sequence analysis. Onderstepoort J Vet Res. 1993;60:307–14.
14. Nel LH, Bingham J, Jacobs JA, Jaftha JB. A nucleotide-specific polymerase chain reaction assay to differentiate rabies virus biotypes in South Africa. Onderstepoort J Vet Res. 1998;65:297–303.
15. Nel L, Jacobs JA, Jaftha JB, von Teichman BF, Bingham J. New cases of Mokola virus infection in South Africa: a genotypic comparison of southern African isolates. Virus Genes. 2000;20:103–6.

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Human African Trypanosomiasis Transmission, Kinshasa, Democratic Republic of Congo

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To investigate the epidemiology of human African trypanosomiasis (sleeping sickness) in Kinshasa, Democratic Republic of Congo, 2 entomologic surveys were conducted in 2005. *Trypanosoma brucei gambiense* and human-blood meals were found in tsetse fly midguts, which suggested active disease transmission. Vector control should be used to improve human African trypanosomiasis control efforts.

Human African trypanosomiasis (HAT) (sleeping sickness) is a parasitic disease caused by a protozoan parasite belonging to the genus *Trypanosoma*. Approximately 60 million persons are exposed to the disease, and 500,000 are currently infected (*1*). HAT has been described as a disease affecting rural areas (*2*). During the recent increase in HAT in historic foci, emergence of foci with new epidemiologic features in urban areas was reported (*3*,*4*). Investigations of these new features showed that development of contiguous relationships between urban areas and surrounding HAT-endemic villages can create conditions favorable for HAT in urban areas (*3*–*5*). Few studies have suggested urban transmission of HAT despite potential epidemiologic consequences of such transmission (*4*).

In Kinshasa, Democratic Republic of Congo, the epidemiologic situation for HAT is complex. In 1903, Dutton-Todd reported a HAT prevalence of 2.4% in apparently healthy inhabitants of Leopoldville (*6*). In 1960, the Kinshasa focus was considered extinct, and no tsetse flies were found in the city. Until 1995, an average of 50 new cases of HAT were reported annually. However, >200 new cases have been reported annually since 1996 (e.g., 443 of 6,205 persons examined in 1998 and 912 of 42,746 persons examined in 1999) (*7*). Ebeja et al. reported that 39% of new cases were urban residents; 60% of them in the first stage of the disease (*3*).

To understand the epidemiology of HAT in this context, several investigations have been undertaken (*3*,*5*,*8*). On the basis of epidemiologic data, some investigators (*3*,*5*) have suggested that urban or periurban transmission of HAT occurs in Kinshasa. However, in a case-control study, Robays et al. concluded that HAT in urban residents of Kinshasa was linked to disease transmission in Bandundu and rural Kinshasa (*8*). To investigate the epidemiology of HAT transmission in Kinshasa, we identified and evaluated contact between humans and flies. The prevalence of *Trypanosoma brucei gambiense* in tsetse fly midguts was determined to identify circulation of this trypanosome between humans and tsetse flies.

The Study

Two entomologic surveys were conducted in 2005 (during the rainy season in February and March and the dry season in June and July) at 8 sites (Ndjili Cecomaf, Ndjili Brasserie, Kimwenza, Mambre, Funa, Buma, Kimbanseke, and Kinkole) in Kinshasa. These sites were selected on the basis of HAT prevalence and entomologic data previously reported (*3*). Rural, periurban, and urban areas (Figure) were defined according to recent mapping of Kinshasa (*5*).

During each survey, tsetse flies were collected in pyramidal traps (*9*), and sex and species were identified. Midguts and blood meals were collected on filter paper, dried, and stored in microtubes. DNA was extracted from midguts or blood meals with 1 mL of 5% Chelex (*10*). Microtubes were incubated for 1 h at 56°C, 30 min at 100°C, and centrifuged for 10 min at 14,000 rpm. The supernatant was collected and used as DNA template for PCR. Blood meals were analyzed according to a method previously described (*11*). Trypanosomes were identified by PCR with specific primers for *T. brucei* s.l. (*12*). and *T. b. gambiense* (*13*).

Because no tsetse flies were found in Buma and Kinkole during the rainy season, these 2 sites were excluded from the second survey (Figure). Entomologic data were analyzed for 610 traps from which data were obtained during the 2 surveys. A total of 897 flies of both sexes were caught; *Glossina fuscipes quanzensis* was the only tsetse fly species found (Table). Fresh midguts containing blood meals or trypanosomes were obtained from 570 living flies. In rural and periurban areas, 54 (9.5%,

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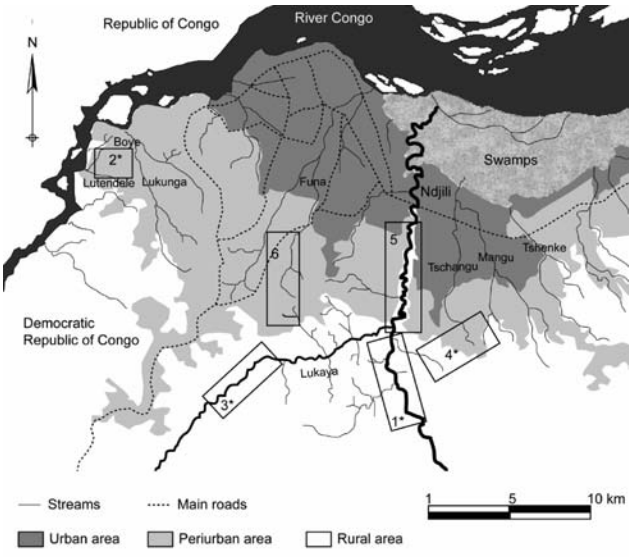

Figure. Map of Kinshasa, Democratic Republic of Congo, showing capture sites where the 2 vector surveys were conducted in urban, periurban, and rural zones. 1, Ndjili Brasseeries; 2, Mambre; 3, Kimwenza; 4, Kimbanseke; 5, Ndjili Cecomaf; 6, Funa. *Capture sites where *Trypanosoma brucei gambiense* was found in tsetse flies.

95% confidence interval [CI] 3.7%–15.2%) teneral flies (young flies that have never taken a blood meal) were identified. Of 570 flies dissected, 117 (20.97%) had sampled blood meals and 110 were successfully identified (Table): 78 meals (67.7%, 95% CI 57.5%–75.9%) were taken from humans and 32 (27.3%, 95% CI 19%–36%) from pigs. PCR identified *T. brucei* s.l. in midguts of 54 (9.5%, 95% CI 7.5%– 11.5%) flies; of these, 54 flies, *T. b. gambiense* was found in 13. The prevalence of *T. b. gambiense* in tsetse midguts was 2.3% (95% CI 1.2%–3.3%). One tsetse fly with a blood meal from a pig was positive for *T. b. gambiense.*

Conclusions

This study confirmed, as reported in previous studies, the presence of *G. f. quanzensis* in Kinshasa (*5*). The most favorable biotopes for tsetse flies are located along the Ndjili, Lukaya, and Boye River valleys. The apparent density per fly was low and similar to the value previously reported (*5*). Pigsties and rivers were the most favorable biotopes for tsetse flies in Kinshasa. Variations in apparent density per trap between biotopes and capture sites are probably linked to climatic factors, the environment surrounding each trap, and urbanization. Increases in human population density attract tsetse flies to rural and periurban areas and concentrate tsetse flies in regions where contact between humans and flies is possible (*14*).

Identification of tsetse flies infected with *T. b. gambiense* confirms contact between flies and patients. The infection rate (2.3%) in our study is comparable to the rates of 1.4% reported in Uganda and 1.9% in Brazzaville, Republic of Congo (*15*). However, *T. b. gambiense* midgut infection is not proof of mature infection, although it shows direct circulation of *T. b. gambiense* between humans and tsetse flies. Infection occurs frequently, as reflected by the feeding preference of *G. f. quanzensis* for humans. This finding shows that urbanization can increase transmission risk by creating conditions that may increase contact between humans and flies, as probably occurred in Kinshasa where we identified a high percentage of human-blood meals (67.7%) and tsetse flies infected with *T. b. gambiense.*

Our results provide evidence for local transmission of HAT in Kinshasa because we detected *T. b. gambiense* midgut infections, human-blood meals, and most urban resident patients in the first stage of the disease. Identification of *T. b. gambiense* infections in flies from different sites indicates transmission in rural and periurban areas. Some patients identified in Kinshasa could have been infected during their movement through areas outside the city for subsistence activities and economic purposes. Local transmission has likely contributed to the increase in HAT in the past decade. The sites of Ndjili Brasserie, Kimwenza, Kimbanseke, and Mambre showed higher risk for HAT transmission. We suggest that vector control be integrated into improved HAT control efforts in urban areas. In Kinshasa, focused vector control activities around

Table. Entomologic results for tsetse flies collected during the dry and rainy seasons, Kinshasha, Democratic Republic of Congo, 2005*

Survey site	No. traps	No. flies	ADT	No. dissected flies	No. teneral flies	No. human-blood meals	No. pig-blood meals	No. *Trypanosoma brucei* s.l.	No. TBG
Ndjili Brasserie	112	423	0.94	244	25	26	18	26	9
Mambre	108	220	0.51	144	13	25	8	12	1
Kimwenza	104	139	0.33	93	6	12	3	8	2
Kimbanseke	118	57	0.12	49	6	9	2	6	1
Ndjili Cecomaf	58	35	0.15	22	3	4	1	–	–
Funa	110	23	0.05	18	1	2	–	2	–
Total	610	897	0.37	570	54	78	32	54	13

*ADT, apparent density per trap; teneral flies, young flies that have never taken a blood meal; TBG, *T. brucei gambien*se group 1.

pigsties and places with water-related activities can reduce fly density, contact between humans and flies, and disease transmission. Contact between tsetse flies and pigs should encourage investigations of the animal reservoir of HAT in Kinshasa. This recommendation is strengthened by the finding of *T. b. gambiense* in a blood meal taken from 1 pig.

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References

1. Control and surveillance of African trypanosomiasis. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser. 1998;881:1–114.
2. Laveissiere C, Sane B, Meda HA. Measurement of risk in endemic areas of human African trypanosomiasis in Côte d'Ivoire. Trans R Soc Trop Med Hyg. 1994;88:645–8.
3. Ebeja AK, Lutumba P, Molisho D, Kegels G. Miaka mia Belinge C, Boelaert M. Sleeping sickness in the region of the town of Kinshasa: a retrospective analysis during the surveillance period 1996–2000. Trop Med Int Health. 2003;8:949–55.
4. Courtin F, Dupont S, Zeze DG, Jamonneau V, Sané B, Coulibaly B, et al. Human African trypanosomiasis: urban transmission in the focus of Bonon (Côte d'Ivoire). Trop Med Int Health. 2005;10:340–6.
5. de Deken R, Sumbu J, Mpiana S, Mansinsa P, Wat'senga F, Lutumba P, et al. Trypanosomiasis in Kinshasa: distribution of the vector, *Glossina fuscipes quanzensis*, and risk of transmission in the peri-urban area. Med Vet Entomol. 2005;19:353–9.
6. Fournet F, Traore S, Cadot E, Hervouet JP. Impact of development of agricultural land on the transmission of sleeping sickness in Daloa, Côte d'Ivoire. Ann Trop Med Parasitol. 2000;94:113–21.
7. Louis FJ, Mia Bilengue CN, Simarro PP. Meso VK, Lucas P, Jannin J. Trypanosomose Humaine Africaine en milieu urbain: une problématique émergente? Bull Soc Pathol Exot. 2003;96:205–8.
8. Robays J, Ebeja AK, Lutumba P, Miaka Mia Belinge C, Kande Betu Ku Mesu V, de Deken R, et al. Human African trypanosomiasis amongst urban residents in Kinshasa: a case-control study. Trop Med Int Health. 2004;9:869–75.
9. Gouteux JP, Lancien J. The pyramidal trap for collecting and controlling tsetse flies (Diptera: Glossinidae). Comparative trials and description of new collecting techniques. Trop Med Parasitol. 1986;37:61–6.
10. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques. 1991;10:506–13.
11. Njiokou F, Simo G, Mbida Mbida JA, Truc P, Cuny G, Herder S. A study of host preference in tsetse fly using a modified heteroduplex PCR based method. Acta Trop. 2004;91:117–20.
12. Moser DR, Cook GA, Ochs DE, Bailey CP, McKane MR, Donelson JE. Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using polymerase chain reaction. Parasitology. 1989;99:57–66.
13. Herder S, Simo G, Nkinin S, Njiokou F. Identification of trypanosomes in wild animals from southern Cameroon using the polymerase chain reaction (PCR). Parasite. 2002;9:345–9.
14. Fournet F, Traoré S, Hervouet JP. Effects of urbanization on the transmission of human African trypanosomiasis in a sub-urban relict forest area of Daloa, Côte d'Ivoire. Trans R Soc Trop Med Hyg. 1999;93:130–2.
15. Rogers A, Kenyabjui EN, Wiggwah AK. A high infection rate of *Trypanosoma brucei* subgroup in *Glossina fuscipes*. Parasitology. 1972;65:143–6.

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Mastomys natalensis and Lassa Fever, West Africa

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PCR screening of 1,482 murid rodents from 13 genera caught in 18 different localities of Guinea, West Africa, showed Lassa virus infection only in molecularly typed *Mastomys natalensis*. Distribution of this rodent and relative abundance compared with *M. erythroleucus* correlates geographically with Lassa virus seroprevalence in humans.

Arenaviruses are emerging in the Americas and Africa and can cause hemorrhagic fevers with case fatalities of up to 15%. These viruses are mainly transmitted through contact with the excreta of their natural hosts, rodents of the family *Muridae*. The Old World arenavirus Lassa virus causes up to 300,000 cases of Lassa fever annually in endemic foci of 2 geographically disjunct regions of West Africa (*1*).

Most arenaviruses have been associated with 1 specific reservoir host species (*2*). Knowledge of the geographic distribution of the taxonomically defined host is therefore essential to understand the epidemiology of human infections. In the 1970s, the rodent host of Lassa virus was classified as *Mastomys natalensis* (*3*); later, when hemoglobin electrophoresis was used for species determination, *M. erythroleucus* and possibly *M. huberti* were also proposed as hosts (*1*). In addition, Lassa virus antigen was detected in *Rattus* and *Mus* genera, raising the possibility that other rodent genera could be involved in transmission (*4*).

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The taxonomy of *Mastomys* is considered unresolved, and species determination remains problematic; 8 distinct species are recognized, and several coexist in Lassa fever–endemic areas (*5*). The uncertainty about their precise natural host relationships with Lassa virus is considered a major obstacle for a better understanding of the restricted distribution of Lassa fever in West Africa (*6*).

In this study, we molecularly typed >1,000 specimens of *Mastomys* spp. from Guinea with a recently established species-specific PCR (*7*). The rodents were then screened for Lassa virus infection with a reverse transcription–PCR (RT-PCR), which was shown to amplify Lassa virus strains from Sierra Leone, Liberia, Guinea, Côte d'Ivoire, and Nigeria, as well as the African arenaviruses Mobala and Ippy and 3 strains of the related lymphocytic choriomeningitis virus (*8*).

The Study

In a survey of rodentborne hemorrhagic fever viruses, 1,591 small mammals were trapped in Guinea from 2002 to 2005. Total RNA was extracted from rodent blood preserved in liquid nitrogen by using the Blood RNA kit (Peqlab, Erlangen, Germany). A 1-step RT-PCR targeting a highly conserved region of the RNA polymerase (L) gene was performed by using primers LVL3359A-plus (5′-AGAATTAGTGAAAGGGAGAGCAATTC), LVL3359D-plus (5′-AGAATCAGTGAAAGGGAAAG CAATTC), LVL3359G-plus (5′-AGAATTAGTGAAAG GGAGAGTAACTC), LVL3754A-minus (5′-CACATC ATTGGTCCCCATTTACTATGATC), and LVL3754D minus (5′-CACATCATTGGTCCCCATTTACTGTGA TC) (Note: Underlined letters represent differences in nucleotides among plus and minus primers.). Because Lassa virus antigen should be frequently detectable in the natural host, and specific antibodies are known to be negatively correlated with its presence, they were not investigated in this study (*9*).

The animals were caught in 18 different study sites representative of the principal geographic regions of Guinea (*10*) (Figure 1). All sites were rural villages with a population ≤1,000. Human Lassa fever seroprevalence was previously reported to be low (0%–11%) in 6 trapping areas and high (25%–55%) in 12 trapping areas (*11–13*, Figure 1). Of 1,482 murid rodents belonging to 13 genera and at least 20 species, we typed 847 as *M. natalensis* and 202 as *M. erythroleucus* but none as *M. huberti*, by using DNA from liver biopsies. In addition, we karyotyped 12 members of the genus *Mastomys* in the field by using standard procedures (*14*).

We obtained positive RT-PCR results from 98 (1.2%) of 1,482 murid rodents. Sequence analysis showed 96 Lassa virus strains with 96%–100% amino acid homology with the prototypic strain Josiah. Lassa virus–positive

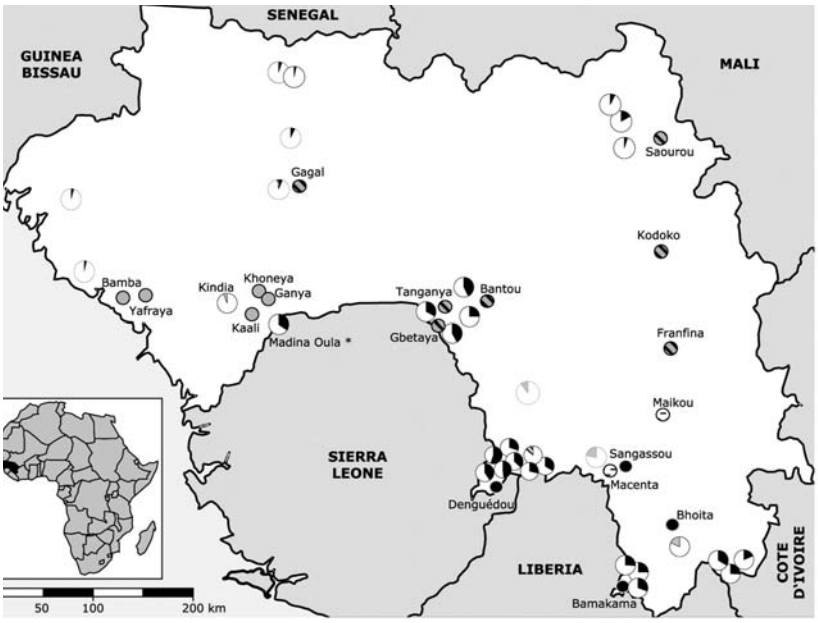

Figure 1. Map of Guinea showing the location of the 18 trapping sites (small circles). Sites where only *Mastomys erythroleucus* or *M. natalensis* were trapped are shaded in gray and black, respectively. Sites where both species were captured are hatched, and sites where no *Mastomys* were caught are marked with a dash. The human Lassa virus seroprevalence in these areas is indicated by the size of the sectors of the larger circles shaded black (*11*), gray (*12*), or hatched (*13*). The asterisk denotes the Madina Oula refugee camp on the border with Sierra Leone, where human seroprevalence was high (*11*), with 23% of the *Mastomys* Lassa virus antigen or antibody positive (*9*). In close localities, no Lassa virus–positive *Mastomys* were found (*9*).

rodents were only captured in the prefectures of Faranah (villages of Gbetaya, Bantou, Tanganya) and Guéckédou (Denguédou), both situated along the border with Sierra Leone (Figure 1). We PCR-typed all Lassa virus–positive rodents unequivocally as *M. natalensis*, with 1 male (no. BA686) additionally confirmed by karyotyping (2n = 32, autosomal fundamental number = 53). Overall, 11.3% of *M. natalensis* were infected with Lassa virus (Table), with 0% in the low seroprevalence area and 5.4%–32.1% in the Lassa fever high seroprevalence area. In the coastal region, where the lowest human Lassa virus seroprevalence (0%–6%) has been reported, only *M. erythroleucus* was

Table. Small mammal species examined for arenavirus infection by reverse transcription PCR*

	Lassa fever high-prevalence areas		Lassa fever low-prevalence areas		Total	
Species	No. examined	No. positive (%)	No. examined	No. positive (%)	No. examined	No. positive (%)
Mastomys natalensis	726	96 (13.2)	121	0	847	96 (11.3)
Mastomys erythroleucus	65	0	137	0	202	0
Mus (Nannomys) spp.	131	1 (0.7)	34	1 (5.6)	165	2 (1.2)
Praomys cf. rostratus	78	0	42	0	117	0
Myomys daltoni	24	0	25	0	49	0
Crocidura spp.	22	0	18	0	40	0
Lophuromys sikapusi	18	0	21	0	39	0
Lemniscomys spp.	19	0	10	0	29	0
Rattus rattus	8	0	51	0	59	0
Tatera cf. guinea	3	0	12	0	15	0
Uranomys ruddi	1	0	6	0	7	0
Hylomyscus simus	5	0	0	0	5	0
Malacomys edwardsi	2	0	0	0	2	0
Hybomys spp.	3	0	0	0	3	0
Mus musculus	0	0	1	0	1	0
Cricetomys gambianus	1	0	0	0	1	0
Graphiurus sp.	1	0	0	0	1	0
Paraxerus sp.	1	0	0	0	1	0
Sylvisorex sp.	1	0	0	0	1	0
Micropteropus sp.	0	0	4	0	4	0
Thamnomys sp.	0	0	1	0	1	0
Dasymys rufulus	0	0	1	0	1	0
Lepus sp.	0	0	1	0	1	0
Total/Lassa virus	1,271	96	320	0	1,591	96 (6)
Total/Arenaviruses	1,271	97	320	1	1,591	98 (6.2)

*The Lassa fever high-prevalence areas comprise the region of Faranah and the forest region (Figure 1); the low-prevalence areas comprise all other localities of the study.

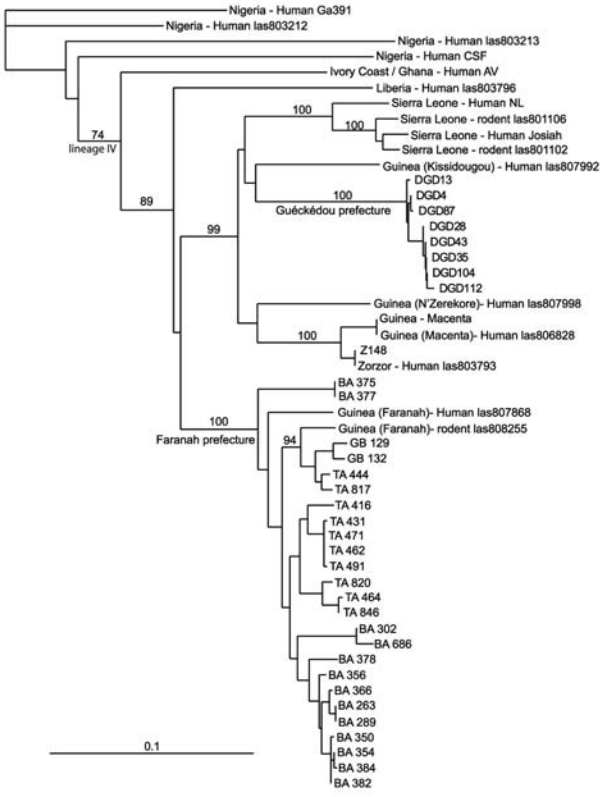

Figure 2. Phylogenetic relationships of Lassa virus strains based on a nucleoprotein gene fragment (631 bp) determined by using the neighbor-joining method. The numbers above branches are bootstrap values >50% (1,000 replicates). Scale bar indicates 10% divergence. Localities are indicated by the specimen label: DGD (Denguédou), BA (Bantou), GB (Gbetaya), and TA (Tanganya).

captured. In contrast, in the forest region, where the highest seroprevalence of up to 55% in selected villages has been found, only *M. natalensis* was trapped (*11*). Both species were captured in the savannah regions, where seroprevalence was 2%–42%, but *M. natalensis* was captured more frequently (Figure 1).

Lassa virus was isolated in cell culture from 32 rodents, and a 631-bp fragment of the nucleoprotein gene previously used for phylogenetic analysis was sequenced (GenBank accession nos. DQ832667–DQ832699) (*15*). The phylogenetic tree shows that all isolates belong to the lineage IV of Lassa virus and that strains from the prefecture of Faranah cluster with strains isolated previously from human patients of the same region (*15*) (Figure 2). We detected 2 novel L-gene sequences that shared 68%–74% homology with lymphocytic choriomeningitis virus in 2 rodents of the *Mus* subgenus *Nannomys.*

Conclusions

Our results indicate that *M. natalensis* is very likely the only reservoir host of Lassa virus in Guinea. Previous studies that identified *M. erythroleucus* and *M. huberti* as reservoirs of Lassa virus in Sierra Leone may have confused the species, especially because *M. natalensis* and *M. huberti* have an identical number of chromosomes (*5*,*7*). Despite a massive trapping effort, *M. huberti* was not detected in the regions of Guinea bordering Sierra Leone that had high human Lassa virus seroprevalence. Therefore, *M. natalensis* is likely also the only reservoir in Sierra Leone. Whether this is also the case for the genetically more remote lineage I–III strains of Lassa virus remains to be evaluated because *M. kollmannspergeri* is also present in Nigeria (*7*,*16*). While it is reassuring that the reservoir for Lassa virus is *M. natalensis*, as reported in the literature since the 1970s, our study demonstrates that proper taxonomic identification of the host is necessary before drawing inferences about the ecology of Lassa virus infection.

The relative abundance of *Mastomys* correlated with human Lassa virus seroprevalence in Guinea: *M. natalensis* was absent from the region with the lowest seroprevalence and was the only *Mastomys* species caught in the highest seroprevalence region. However, if both *M. natalensis* and *M. erythroleucus* were present, human Lassa virus prevalence was either low, intermediate, or high (Figure 1). This is a novel finding, which confirms and expands on 2 previous studies conducted in Guinea that reported a correlation between the absence of *Mastomys* spp. and a low human Lassa virus seroprevalence (*9*,*12*).

The results of this study have multiple implications for explaining the patchy occurrence of Lassa virus in Guinea and neighboring countries, as well as for Lassa fever control. First, assuming that *M. natalensis* is the only host of Lassa virus, natural nidality may occur in a similar fashion as that described for Bolivian hemorrhagic fever caused by Machupo virus. The Machupo virus reservoir host, *Callomys callosus*, has been shown by genetic analysis to be paraphyletic. The populations of rodents responsible for the maintenance and transmission of Machupo virus were monophyletic lineages different from *C. callosus* in nondisease–endemic regions of Bolivian hemorrhagic fever and coevolving with the virus (*17*). *M. natalensis* specimens from regions with high and low Lassa virus activity are being genetically investigated to determine lineages and population structure. Second, the geographic region of potential rodent-to-human transmission of lineage IV strains of Lassa virus is most likely defined by the occurrence of *M. natalensis*. *Mastomys* collections from Sierra Leone and Liberia could be molecularly retyped to reevaluate areas of potential Lassa fever reemergence. Third, our mass screening for arenaviruses found only Lassa virus in *Mastomys* and a novel lymphocytic choriomeningitislike virus in *Mus* spp. of the subgenus *Nannomys*, which are closely related to *Mus musculus*, the Eurasian host of lymphocytic choriomeningitis virus. These findings support

the hypothesis of a species-specific association of arenaviruses with their rodent hosts, resulting in cophylogeny.

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References

1. McCormick JB, Webb PA, Krebs JW, Johnson KM, Smith ES. A prospective study of the epidemiology and ecology of Lassa fever. J Infect Dis. 1987;155:437–44.
2. Salazar-Bravo J, Ruedas LA, Yates TL. Mammalian reservoirs of arenaviruses. Curr Top Microbiol Immunol. 2002;262:25–63.
3. Monath TP, Newhouse VF, Kemp GE, Setzer HW, Cacciapuoti A. Lassa virus isolation from *Mastomys natalensis* rodents during an epidemic in Sierra Leone. Science. 1974;185:263–5.
4. Wulff H, Fabiyi A, Monath TP. Recent isolations of Lassa virus from Nigerian rodents. Bull World Health Organ. 1975;52:609–12.
5. Granjon L, Duplantier J-M, Catalan J, Britton-Davidian J. Systematics of the genus *Mastomys* (Thomas, 1915) (Rodentia: Muridae). A review. Belg J Zool. 1997;127(Suppl. 1):7–18.
6. Mills JN, Childs JE. Ecologic studies of rodents reservoirs: their relevance for human health. Emerg Infect Dis. 1998;4:529–37.
7. Lecompte E, Brouat C, Duplantier JM, Galan M, Granjon L, Loiseau A, et al. Molecular identification of four cryptic species of *Mastomys* (Rodentia, Murinae). Biochem Syst Ecol. 2005;33:681–9.
8. Vieth S, Drosten C, Lenz O, Vincent M, Omilabu S, Hass M, et al. A reverse transcription PCR assay for detection of Lassa virus and related Old World arenaviruses targeting the L gene. Trans R Soc Trop Med Hyg. In press.
9. Demby AH, Inapogui A, Kargbo K, Koninga J, Kourouma K, Kanu J, et al. Lassa fever in Guinea: II. Distribution and prevalence of Lassa virus infection in small mammals. Vector Borne Zoonotic Dis. 2001;1:283–96.
10. White F. 1983. The vegetation of Africa; a descriptive memoir to accompany the UNESCO/AETFAT/UNSO vegetation map of Africa. Paris: UNESCO; 1983.
11. Lukashevich LS, Clegg JC, Sidibe K. Lassa virus activity in Guinea: distribution of human antiviral antibody defined using enzyme-linked immunosorbent assay with recombinant antigen. J Med Virol. 1993;40:210–7.
12. Bausch DG, Demby AH, Coulibaly M, Kanu J, Goba A, Bah A, et al. Lassa fever in Guinea: I. Epidemiology of human disease and clinical observations. Vector Borne Zoonotic Dis. 2001;1:269–81.
13. ter Meulen J, Lukashevich I, Sidibe K, Inapogui A, Marx M, Dorlemann A, et al. Hunting of peridomestic rodents and consumption of their meat as possible risk factors for rodent-to-human transmission of Lassa virus in the Republic of Guinea. Am J Trop Med Hyg. 1996;55:661–6.
14. Ford CE, Hamerton JL. A colchicine, hypotonic citrate, squash sequence for mammalian chromosomes. Stain Technol. 1956;31:247–51.
15. Bowen MD, Rollin PE, Ksiazek TG, Hustad HL, Bausch DG, Demby AH, et al. Genetic diversity among Lassa virus strains. J Virol. 2000;74:6992–7004.
16. Robbins CB, Van der Straeten E. Comments on the systematics of *Mastomys* Thomas 1915 with a description of a new west African species (Mammalia: Rodentia: Muridae). Senckenbergiana Biologica. 1989;69:1–14.
17. Salazar-Bravo J, Dragoo JW, Bowen MD, Peters CJ, Ksiazek TG, Yates TL. Natural nidality in Bolivian hemorrhagic fever and the systematics of the reservoir species. Infect Genet Evol. 2002;1:191–9.

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Zoonotic Focus of Plague, Algeria

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After an outbreak of human plague, 95 *Xenopsylla cheopis* fleas from Algeria were tested for *Yersinia pestis* with PCR methods. Nine fleas were definitively confirmed to be infected with *Y. pestis* biovar orientalis. Our results demonstrate the persistence of a zoonotic focus of *Y. pestis* in Algeria.

Yersinia pestis, the agent of plague, has shaped the course of human history, killing millions of people in 3 major pandemics (*1*). This bacterium remains endemic in parts of Asia, Africa, and the Americas, where it poses a substantial zoonotic threat to human populations. The organism has also recently received attention as a possible bioterrorism agent (*2*). *Y. pestis* primarily infects small mammals, particularly rodents, and is transmitted from infected to uninfected hosts by fleas (*1*). More than 200 different mammalian species and at least 80 different species of fleas have been implicated in maintaining *Y. pestis* in zoonotic foci throughout the world (*1*,*3*). Among them, the rat flea, *Xenopsylla cheopis*, is considered a major competent vector (*1*).

The Study

In June 2003, an outbreak of plague emerged in the Oran area of Algeria (*4*). During the following weeks, a total of 11 confirmed and 7 suspected cases of plague were reported from the same area (*4*). The University Hospital in Oran confirmed the plague diagnosis. All cases were bubonic plague; septicemia and coma later developed in 2 patients. According to national health records, the last outbreak in Oran was in 1946 and the last human cases of plague occurred in Algeria in 1950. The aim of this study was, by using molecular methods, to investigate the presence of *Y. pestis* in fleas collected from rodents.

The sites of the original focus of reported plague cases were Kehailia (35°29′N, 0°32′E) and Benaouali (35°33′N, 0°21′E), in the area of Oran and Mascara, ≈450 km west of the capital, Algiers (Figure). Fleas were collected from rodents trapped inside human residences and peridomestic areas within this area (Figure) from September 2004 to May 2005 by using BTS (Besancon Technique Service, INRA, Montpellier, France) and Sherman Trap (H.P. Sherman Traps, Tallahassee, FL, USA). Specimens were stored in absolute ethanol before being tested in Marseille, France, in May 2005. Preliminary morphologic identification was performed (by I.B.) by using entomologic taxonomic keys (*5*). Identification was confirmed by sequencing regions of siphonapteran 18S rDNA, as previously described (*6*). Sequences were compared with flea sequences deposited in the 18S rDNA database of the Whiting Laboratory (*6*). Ethanol-preserved fleas were rinsed with distilled water for 10 minutes and dried on sterile filter paper in a laminar biosafety hood. Fleas were crushed individually in sterile Eppendorf tubes with the tips of a sterile pipette. DNA was extracted by using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *Y. pestis* DNA was detected by real-time PCR with primers against the plasminogen activator (*Pla*) gene of *Y. pestis* (Eurogentec, Angers, France) as previously described (*7*). For this assay, negative controls consisted of extracted DNA of uninfected fleas from colonies of our laboratory. Positive control consisted of a plasmid previously developed in our laboratory for detecting bioterrorism agents; using this control permitted both control of cycling efficacy and detection of contamination during the PCR process (*7*). To confirm positive results, extracted DNA was amplified, and PCR products were sequenced by using 2 alternative spacer targets of *Y. pestis (*spacers YP8 and YP9) as previously described (*8*). Positive sample products were sequenced with an ABI 3130Xl Genetic Analyzer (Applied Biosystems, Coignieres, France). Sequences were compared with those available in GenBank by using the nucleotide-nucleotide BLAST (blastn) program (available

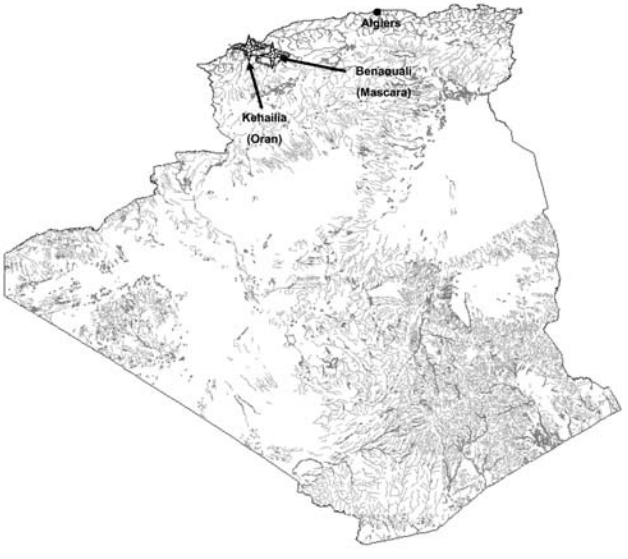

Figure. Map of the zone where fleas were collected and sites of epidemic plague reported, Algeria, June 2003.

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from http://www.ncbi.nlm.gov/BLAST/) together with those of our local database (*8*).

Ninety five fleas were collected from rodents, including 21 *Rattus rattus*, 13 *R. norvegicus*, 7 *Mus musculus*, and 8 *M. spretus* trapped inside the houses and in the peridomestic areas of the cities of Kehailia and Benaouali (Figure). Using taxonomic keys, we identified all 95 fleas morphologically as *X. cheopis* (the rat flea); fleas index was calculated according to rats or mice, respectively (3.333 and 3.125). Identity was confirmed by sequencing and comparison of an 1,867-bp informative region of siphonapteran 18S rDNA. (*6*). Using the LightCycler (LC, Roche Diagnostics, Mannheim, Germany) real-time-PCR assay previously developed for detecting bioterrorism agents targeting the plasminogen activator gene (*7*), we found 20 (21.05%) of 95 fleas positive with a cycle threshold (Ct) value ranging from 27.2 to 33.91. Among these 20 positive fleas, 9 were also positive in multiple spacer typing (MST) assays by using primers targeting spacer YP8 and 8 with primers targeting the spacer YP9 (Table). No nucleic acids were amplified from the negative controls. The mean Ct value obtained with the LC assay for the 9 fleas positive with the YP8 primers was significantly lower that the mean Ct value for the remaining 11 fleas only positive with the LC assay (29.56 ± 1.55; $n = 9$ vs. 31.98 ± 1.13; $n = 11$; $p = 0.0005$) (Table). Thus, LC assay appears to be more sensitive than MST assay. Sequences of the PCR products obtained with YP8 and YP9 primers were 100% identical to sequences of *Y. pestis* biovar *orientalis* (GenBank accession nos. AE017139 and YP02648) (*8*).

Conclusions

In this study we present molecular evidence of *Y. pestis* in 20 *X. cheopis* fleas collected in the area of Oran, Algeria. The molecular methods used in our study have been previously validated (*7*,*8*), and precautions were taken to reduce risks for contamination during processing.

Rieux, the hero of Albert Camus (*9*) in "La Peste," aimed to relate the events of the plague outbreak in Oran in the 1940s with the highest objectivity. He stated that "the virus" of plague can come back 1 day and he asked to be aware when it did. Apparently plague has retired but is waiting in numerous foci and could reemerge, as it did in India during the 1990s. The "comeback" of plague in the region of Oran occurred in June 2003. In this study, we detected *Y. pestis* in rodent fleas collected from September 2004 to May 2005 in the same area as those plague cases occurred. Our results confirm that *Y. pestis* infection is still present in Algeria. The persistence of zoonotic foci of plague is worrying since persons living in these areas remain in close contact with rodents and fleas. Despite the absence of new cases since June 2003, the risk for further outbreaks remains high. Surveillance should be maintained to monitor this natural focus and potential spread resulting from climatic or habitat influences (*10*). A strong case could be made to extend surveillance to adjacent countries such as Libya and Mauritania, which also have natural foci of plague, according to the World Health Organization. In conclusion we believe that detection of *Y. pestis* in fleas can be a useful tool for epidemiologic surveillance of plague in specific settings and could thus serve to study the risk for reemergence of the disease.

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References

1. Pollitzer R. Plague. WHO monograph series no. 22. Geneva: World Health Organization; 1954.
2. Riedel S. Plague: from natural disease to bioterrorism. Proc (Bayl Univ Med Cent). 2005;18:116–24.
3. Poland J, Barnes AM. Plague. In: Steele JH, editor. CRC handbook series in zoonoses. Boca Raton (FL): CRC; 1979. p. 515–97.
4. World Health Organisation. Plague in Algeria – update 2. Wkly Epidemiol Rec. 2003;78:253–60.
5. Beaucournu JC, Launay F. [Les puces (*Siphonaptera*) de France et du bassin méditerranéen occidental]. Paris: Fédération Française des Sociétés de Sciences Naturelles; 1990.
6. Bitam I, Parola P, Dittmar de la Cruz K, Matsumoto K, Baziz B, Rolain JM, et al. First molecular detection of *Rickettsia felis* in fleas from Algeria. Am J Trop Med Hyg. 2006;74:532–5.

Table. Identification and biologic source of *Yersinia pestis* isolates*

Real-time PCR (LC)	Mean Ct value ± SD (LC)	MST (YP8)	MST (YP9)	No. fleas
+	29.56 ± 1.55†	+	+	8
+	30.25	+	–	1
+	31.98 ± 1.13†	–	–	11
–	ND	–	–	75

*Examined by LightCycler (LC) and multiple spacer typing (MST) assays. Ct, cycle threshold; SD, standard deviation; ND, not done.
†$p<0.05$ between mean Ct values of fleas positive with LC assay only and fleas positive with MST and LC assays.

7. Charrel RN, La Scola B, Raoult D. .Multi-pathogens sequence containing plasmids as positive controls for universal detection of potential agents of bioterrorism. BMC Microbiol. 2004;4:1–11
8. Drancourt M, Roux V, Dang LV, Tran-Hung L, Castex D, Chenal-Francisque V, et al. Genotyping, Orientalis-like *Yersinia pestis*, and plague pandemics. Emerg Infect Dis. 2004;10:1585–92.
9. Camus A. La peste. Paris: Gallimard Poche; 1996.
10. Duplantier JM, Duchemin JB, Chanteau S, Carniel E. From the recent lessons of the Malagasy foci towards a global understanding of the factors involved in plague reemergence. Vet Res. 2005;36:437–53.

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Rabies Virus Maintained by Dogs in Humans and Terrestrial Wildlife, Ceará State, Brazil

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Rabies viruses circulating in Ceará, Brazil, were identified by molecular analysis to be related to variants maintained by dogs, bats, and other wildlife. Most of these viruses are associated with human rabies cases. We document the emergence of a rabies virus variant responsible for an independent epidemic cycle in the crab-eating fox (*Cerdocyon thous*).

After dog rabies control programs were implemented in Ceará State, Brazil, a the number of human cases decreased (*1*,*2*). Thereafter, the epidemiologic importance of rabies in wildlife became evident. From 1990 through 2005, a total of 173 cases of rabies were reported in *Cerdocyon thous (*crab-eating fox*)*, 25 in *Callithrix. j. jacchus (*common marmoset*)* and 6 in *Procyon cancrivorous* (crab-eating raccoon). During this period, in 13 of 40 human cases reported in Ceará, wildlife was the source of infection (*2*).

In 1996, because of this new epidemiologic situation, public health authorities launched an educational program, and no human cases due to wildlife were recorded in 1999, despite 84 cases in wildlife registered that year (*2*). The objective of this study was to elucidate some of the epidemiologic events involved in rabies emergence among wildlife in Ceará.

The Study

We studied 22 samples, from dogs, cattle, wildlife, and humans in Ceará, obtained from 1997 to 2003 (Table). Samples were antigenically characterized by using a monoclonal antibody (MAb) panel against the viral nucleoprotein (*3*–*5*). Isolates were injected into the brains of suckling mice, and brain impressions were made for MAb typing (*3*,*5*). Characterization of the samples identified 4 antigenic variants. Antigenic variant-2 (AgV2), maintained by dogs, was found in all *C. thous*, *P. crancrivourous,* and human cases and in all dog isolates with the exception of brdg5360, which was positive with all the MAbs. Antigenic variant-3 (AgV3), epidemiologically associated with vampire bats, *Desmodus rotundus*, was identified in 3 bovine samples. A previously reported profile, representing an AgV that circulates in marmosets in Ceará (*5*), was detected in sample brsg5696.

The Ceará viruses were analyzed genetically through a comparative phylogenetic study based on a 320-bp fragment of the nucleoprotein gene, from position 1157 to 1476, as compared with SADB19 (*5*–*7*,*8*). These isolates were also compared with rabies virus variants circulating among domestic animals and wildlife from the Americas. The viral RNA was extracted from infected tissues, and the cDNA was obtained by reverse transcription–PCR techniques, using primers 21 g and 304, and was sequenced with primer 304 (*7*,*9*). The phylogenetic analyses were made by using the PileUp program of the Wisconsin Package Version 10.1 (*10*) and the programs DNADIST, NEIGHBOR, SEQBOOT, and CONSENSE of the PHYLIP package (*11*). The expressed percentages of identity refer to the nucleotide sequences. The trees were obtained with the TREEVIEW program (*12*). The phylogenetic analyses showed segregation in 5 lineages, A–E (Figure 1), which was statistically supported by high bootstrap values.

Lineage A was represented by a sample from a dog from Maranguape, which was obtained in 2001. This virus showed its closest genetic relationship with lineage B (identity 92.4%–94.2%). Lineage B was formed by all the *C. thous* isolates, a sample from a human bitten by a *P. cancrivorous* raccoon in Fortaleza in 1997, and a virus from a *P. crancrivorous* raccoon collected in Maranguape during 2001 (intrinsic identity 96.5%–100%). This lineage showed its highest percentage of identity with lineage C (intrinsic identity 90.6%–92.8%). Lineage C consisted of 9 human samples collected in 5 different counties from 2000 to 2003 and an isolate obtained from a dog in 2000. The samples were highly homologous (intrinsic identity 99.1%–100%). Lineage D included 3 bovines collected in 3 geographically distant counties during 2000 and 2001 (intrinsic identity 97.5%–98.4%). Lineage E was represented by the only sample collected from a *C. j jacchus* marmoset, These last 2 lineages were related distantly to all the others.

When compared with representatives of rabies variants maintained by terrestrial and bat species in the Americas

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Table. Identification, origin, and antigenic and genetic variant of 22 rabies virus samples isolated from Ceará State, Brazil

Identification	Animal species	Year of isolation	Origin	Antigenic variant	Genetic variant	Group	GenBank accession no.
Brhm4531	Human	1997	Fortaleza	AgV2	Dog	B	DQ447947
Brcth4122	*Cerdocyon thous*	1998	-	AgV2	Dog	B	DQ447948
Brhm5325	Human	2000	Caucaia	AgV2	Dog	C	DQ447949
Brdg5360	Dog	2000	Caucaia	All+	Dog	C	DQ447950
Brcth5361	*C. thous*	2000	Paracuru	AgV2	Dog	B	DQ447951
Brbv5339	Bovine	2000	Antonina do Norte	AgV3	Vampire bat	D	DQ447952
Brbv5374	Bovine	2000	Quixere	AgV3	Vampire bat	D	DQ447953
Brhm5691	Human	2001	Caucaia	AgV2	Dog	C	DQ447954
Brdg5693	Dog	2001	Maranguape	AgV2	Dog	A	DQ447955
Brcth5695	*C. thous*	2001	Barroquinha	AgV2	Dog	B	DQ447956
Brcth5697	*C. thous*	2001	Caninde	AgV2	Dog	B	DQ447957
Brcth5692	*C. thous*	2001	Maranguape	AgV2	Dog	B	DQ447958
Brpcr5698	*P. cancrivorous*	2001	Maranguape	AgV2	Dog	B	DQ447959
Brbv5694	Bovine	2001	Aquiraz	AgV3	Vampire bat	D	DQ447960
Brsg5696	*C. j. jacchus*	2001	Caucaia	AgV new*	Marmoset	E	DQ447961
Brhmu138	Human	2002	Fortaleza	AgV2	Dog	C	DQ447962
Brhmu142	Human	2003	Fortaleza	AgV2	Dog	C	DQ447963
Brhmu130	Human	2003	Umirim	AgV2	Dog	C	DQ447964
Brhmu146	Human	2003	Fortaleza	AgV2	Dog	C	DQ447965
Brhmu145	Human	2003	Tururu	AgV2	Dog	C	DQ447966
Brhmu129	Human	2003	Fortaleza	AgV2	Dog	C	DQ447967
Brhmu131	Human	2003	Maracanau	AgV2	Dog	C	DQ447968

*Pattern related to isolates from marmosets.

(Figure 2), lineages A, B, and C continue to segregate as independent lineages with high statistical support. Ceará bovine samples representing lineage D clustered with *D. rotundus* and *D. rotundus*–related cases from Latin America (intrinsic identity 94%–97.8%).

The only sample representative of lineage E segregated with 2 isolates from humans bitten by *C. j. jacchus* and a sample collected from a marmoset kept as a pet (*5*). The isolates were highly homologous to each other (intrinsic identity 98.7%–100%).

Conclusions

A thorough description of rabies epidemiology depends on a comprehensive surveillance program and application of accurate molecular methods to discriminate among different variants and the emergence of new foci. Antigenic and limited sequencing analyses were used to better understand the emergent epidemiologic events in wildlife in Ceará, Brazil. These analyses allowed identification of 5 potential cycles in this region, despite antigenic homogeneity.

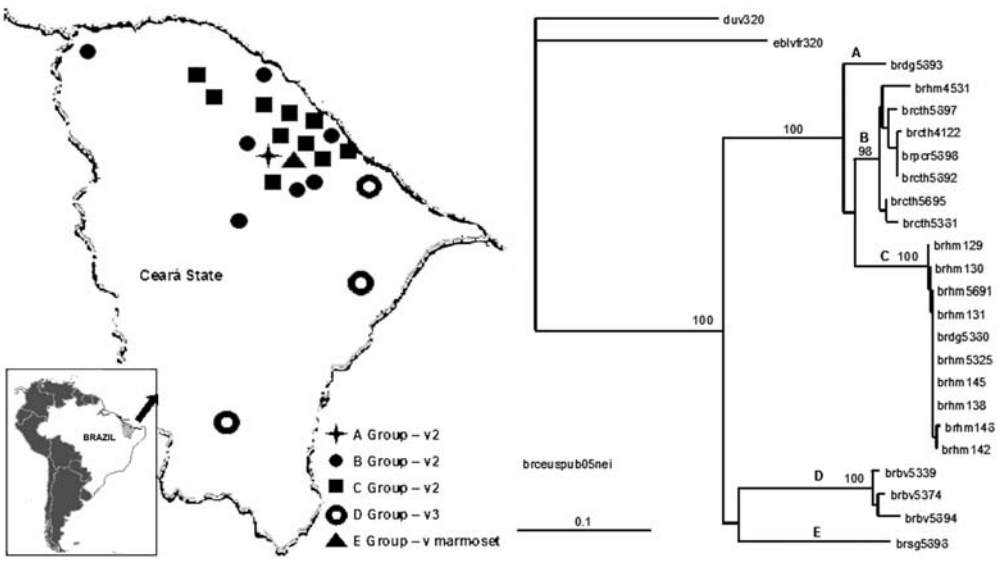

Figure 1. Rabies virus isolates by geographic localization and neighbor-joining tree showing a comparison of the groups formed by Ceará State, Brazil, samples isolated from 1997 to 2003. Bootstrap values of >50% obtained from 100 resamplings of the data using distance matrix methods are shown in the nodes.

Lack of antigenic and genetic relationships of sample brdg5693, representing lineage A, with the rest of the isolates from Ceará and the known terrestrial rabies vectors from the Americas shows that this virus is a variant not previously described. This virus was geographically and temporarily associated with samples brpcr5698 and brcth5692, obtained in Maranguape during 2001. These circumstances demonstrated the existence of at least 2 overlapping endemic cycles in this area. Lineage B was formed mainly by isolates from *C. thous*, which indicates the existence of an emerging rabies cycle in this species.

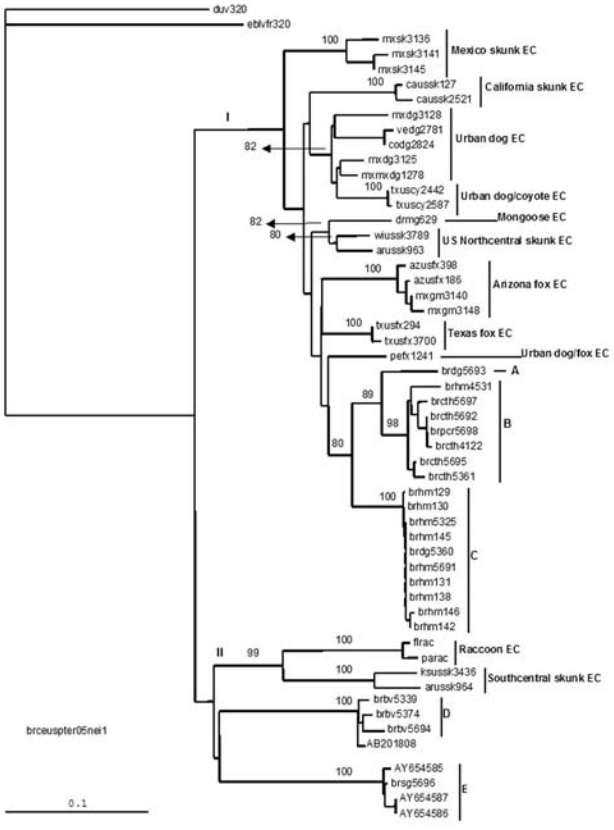

Figure 2. Neighbor-joining tree showing a comparison of Ceará samples (groups A, B, C, D, E) with isolates obtained from the Americas. Bootstrap values of >50% obtained from 100 resamplings of the data using distance matrix methods are shown in the nodes. The sequences from Latin America used in the comparison were identified as as follows: group I, dogs and terrestrial wildlife from Mexico, Venezuela, Colombia, Dominican Republic, and Peru (mxsk, skunk from Mexico; mxdg and mxmx, dog from Mexico; vedg, dog from Venezuela; codg, dog from Colombia; drmg, mongoose from Dominican Republic; mxgm, bobcat from Mexico; pefx, fox from Peru) and terrestrial wildlife from the United States (caussk, skunk from California; txuscy, coyote from Texas; wiussk, striped skunk from Wisconsin; arussk, striped skunk from Arkansas; azusfox, gray fox from Arizona; txusfx, gray fox from Texas); and group II, terrestrial wildlife from the United States (flrac, raccoon from Florida; parac, raccoon from Philadelphia; ksussk, striped skunk from Kansas; arussk, striped skunk from Arkansas). The antigenic variant and endemic cycle to which it belongs are shown in the tree. (GenBank accession no. AB201803 is a vampire bat from Brazil and nos. AY654585, AY654587, AY654586 are humans and a marmoset from Brazil). EC, endemic cycle.

The epidemiologic situation in Ceará was complicated because of overlapping distributions of dog and *C. thous* rabies cases (Figure 1). Tree topology and genetic relationships between dog and *C. thous* variants suggested that the canine virus was introduced in *C. thous* populations because of spillover events, which gave rise to an emergent cycle. A similar event was described between domestic dogs and *Canis adustus* (jackal) in Zimbawe. In this case, the variant circulating in dogs was introduced into the *C. adustus* population by spillover events, with the consequent emergence of an independent cycle (*13*). Recently, the hoary fox has been identified as a rabies reservoir in Brazil (*14*).

Inclusion in lineage B of an isolate obtained from a human bitten by a *P. cancrivorous* raccoon and another sample collected from this species suggested the risk of establishing *C. thous* variant in *P. cancrivorous*. The niches of these 2 species overlap, which facilitates their encounters. Additional surveillance is necessary to clarify this situation.

Epidemiologic data which indicates that humans had been exposed to dog bites, results of molecular characterization, and inclusion of a dog isolate in the C lineage strongly incriminate the dog as the reservoir of this variant. Identification of the source of infection by using classic surveillance alone is complicated by the presence of multiple cycles of transmission. Genetic comparison of samples from lineage D with viruses representing bats viruses from the Americas helped to identify *D. rotundus* as the source of livestock infection.

The close genetic relationship of sample brsg5696 with rabies isolates obtained from *C. j. jacchus* and human cases bitten by marmosets further supported *C. j. jaccuss* as the most important vector of this variant. This finding indicates that this species plays an important role for disease maintenance in nature.

Methods for antigenic and genetic identification of rabies samples isolated in the Americas have contributed effectively to the development of health programs, as well as recognition of possible wild reservoirs of urban rabies. The emergence of new cycles in Latin American wildlife indicates the need to strengthen surveillance programs in these species and research development for the evaluation of the feasibility of oral vaccination interventions.

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References

1. Pan-American Health Organization/World Health Organization. Prevention and control of diseases. Veterinary Public Health Unit. Elimination of the human rabies transmitted by dogs in Latin America: analysis of the situation, 2004. Washington: Pan American Health Organization; 2005. vol. 37, p. 10–29.
2. Núcleo de Controle das Endemias Transmissíveis por vetores—NUEND, Coordenadoria de Apoio ao Desenvolvimento da Atenção à Saúde—CODAS, 1998–2003. Boletim da Secretaria da Saúde do Estado do Ceará, Brasil, 2004.
3. Favoretto SR, Carrieri ML, Cunha EMS, Aguiar EAC, Silva LHQ, Sodré M, et al. Antigenic typing of Brazilian rabies virus samples isolated from animals and humans, 1989–2000. Rev Inst Med Trop Sao Paulo. 2002;44:91–5.
4. Pan-American Health Organization/World Health Organization. Los anticuerpos monoclonales en la caracterización y vigilancia de los virus de la rábia en América Latina y el Caribe. Pan American Journal of Public Health. 2000;8:214–7.
5. Favoretto SR, Mattos CC, Morais NB, Araújo FAA, Mattos CA. Rabies in marmosets (*Callithrix jacchus*) from the state of Ceará, Brazil. Emerg Infect Dis. 2001;7:1062–5.
6. Conzelmann KK, Cox JK, Schneider LG, Thiel HJ. Molecular cloning and complete sequence of the attenuated rabies virus SABB19. Virology. 1990;175:485–99.
7. Smith JS. Rabies virus. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken R, editors. Manual of clinical microbiology, 6th ed. Washington: American Society for Microbiology Press; 1995. p. 997–1003.
8. de Mattos CC, de Mattos CA, Loza-Rubio E, Aguilar-Setién A, Orciari LA, Smith JS. Molecular characterization of rabies virus isolates from Mexico: implications for transmission dynamics and human risk. Am J Trop Med Hyg. 1999;61:587–97.
9. de Mattos CA, de Mattos CC, Smith JS, Miller E, Papo S, Utrera A, et al. Genetic characterization of rabies field isolates from Venezuela. J Clin Microbiol. 1996;34:1553–8.
10. Version WP. 10.1 Madison (WI): Genetics Computer Group; 2000.
11. Felsenstein, J. Phylip: Phylogeny inference pakage. Version 3,5c. Seattle (WA): University of Washington;, 1993.
12. Page, Ram. D. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applied Biocience. 1996;12:357–8.
13. Bingham J, Foggim CM, Wandeler AI, Hill FW. The epidemiology of rabies in Zimbabwe. 2. Rabies in jackals (*Canis adustus* and *Canis mesomelas*). Onderstepoort J Vet Res. 1999;1:11–23.
14. Bernardi F, Nadin Davis SA, Wandeler AI, Armstrong J, Gomes A, Lima FS, et al. Antigenic and genetic characterization of rabies viruses isolated from domestic and wild animals of Brazil identifies the hoary fox as a rabies reservoir. J Gen Virol. 2005;86:3153–62.

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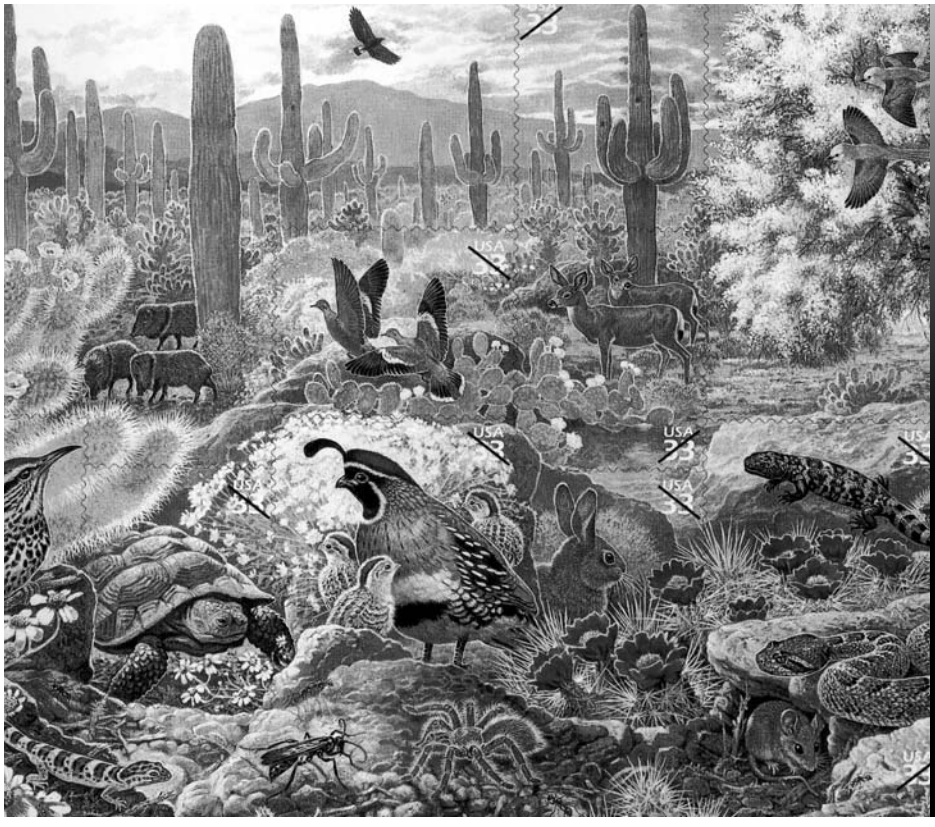

West Nile Virus Antibody Prevalence in Wild Mammals, Southern Wisconsin

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Twenty percent prevalence of West Nile virus antibody was found in free-ranging medium-sized Wisconsin mammals. No significant differences were noted in antibody prevalence with regard to sex, age, month of collection, or species. Our results suggest a similar route of infection in these mammals.

In 1999, West Nile virus (WNV) was detected for the first time in the United States in dead American crows (*Corvus brachyrhynchos)*, and a disease surveillance system that used dead crows was established (*1*,*2*). Serologic surveys to determine the prevalence of WNV exposure in free-ranging mammals (*3*–*6*) are relatively rare. Although WNV can infect a wide range of vertebrates, mammals are assumed to be dead-end hosts (*7*). We report the results of a 2003–2004 WNV serosurvey in medium-sized mammals from south-central Wisconsin.

The Study

We obtained samples from a part of south-central Wisconsin (Dane and Iowa Counties) recently identified as an area where white-tailed deer (*Odocoileus virginianus*) had chronic wasting disease infection (*8*). Medium-sized free-ranging mammals were collected as part of a larger study to evaluate the potential for transmission of chronic wasting disease from infected white-tailed deer carcasses to scavenging mammals. A total of 228 medium-sized mammal carcasses, consisting of 78 raccoons (*Procyon lotor)*, 71 Virginia opossums (*Didelphis virginiana*), 59 coyotes (*Canis latrans*), 7 red foxes (*Vulpes vulpes*), 6 striped skunks (*Mephitis mephitis*), 5 feral cats (*Felis catus*), and 2 badgers (*Taxidea taxus)*, were obtained by trapping, shooting, or collecting fresh road kills during October 2003 through April 2004. These animals were collected in rural areas consisting of small woodlots, agricultural fields, and roadsides.

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Blood samples from the carcasses were collected by absorbtion into Nobuto strips (Toyo Roshi Kaisha, Ltd, Tokyo, Japan), labeled, air dried, and stored at ambient temperature until submitted to the National Wildlife Health Center (NWHC). A 1:20 serum dilution was prepared in the laboratory by following the manufacturer's instructions for extraction from the Nobuto strip. The dilution was stored at 0°C until it was tested.

Before testing, serum samples were heat inactivated (56°C for 30 min) to eliminate any nonspecific virus inhibitors. Serum controls were included for each sample to determine whether any individual serum sample was toxic to the cell culture used. The samples were screened for WNV antibody against 100 PFU by using the plaque reduction neutralization test (PRNT) (*9*). The WNV used was isolated by NWHC in September 1999 from the spinal cord, sciatic nerve, and brain pool of an American crow found dead in the state of New York (strain NY99–35261 –11). Serum samples were considered to be positive for flavivirus antibody if they neutralized ≥50% of the WNV test dose at a serum dilution ≥1:40. Positive serum samples were subsequently titered by PRNT (*9*) against both WNV and Saint Louis encephalitis virus (SLEV) to determine antibody titer and specificity. The SLEV strain (TBH-28 ASFL) was obtained from the Centers for Disease Control and Prevention, Atlanta, Georgia. Serum antibody titers were determined by attempting to neutralize WNV and SLEV using 2-fold serial dilutions ranging from 1:20 to 1:2,560. The serum titer endpoint was considered to be that dilution ≥1:40 still capable of neutralizing ≥90% of the virus test dose. The antibody titer of each serum against the 2 viruses was compared. Serum samples were considered positive for WNV antibody if the titer was ≥4-fold more than the serum titer against SLEV. If a ≤2-fold SLEV and WNV titer difference was noted, the serum antibody was considered to be due to exposure to a previously described or not yet recognized flavivirus.

Conclusions

In 2001 the Wisconsin Department of Health and Family Services (DHFS) reported the first isolation of WNV from a crow (DHFS, unpub. data), and surveillance for the virus was initiated throughout Wisconsin. By 2003, WNV was detected throughout Wisconsin (including our sampling area); most positive corvid cases coincided with our sampling period from late summer to fall. The Wisconsin Department of Natural Resources reported (http://www.dnr.state.wi.us) that WNV had been detected in 145 (48%) of 301 dead American crows and 17 (22%) of 77 dead blue jays (*Cyanocitta cristata*) tested. Most of these positive avian cases were detected from mid-August through October. WNV was also detected in 70 of 72 Wisconsin counties, including the 2 in our study.

Our data indicate that the mammals tested in 2003 and 2004 were more likely to be exposed to WNV than to other flaviviruses. Of the 228 medium-sized mammals tested, 70 (31%) (Table) had flavivirus antibody, with specific WNV antibody in 46 (66%) of 70. Because the numbers of samples were insufficient, we could not evaluate WNV antibody prevalence in red foxes, striped skunks, feral cats, or badgers. In the raccoons, opossums, and coyotes, exposure to a flavivirus was detected in 69 (33%) of 208 and specific WNV antibody in 45 (66%) of 68. Because of variation in sample quality from carcasses obtained by different means, our results may provide a conservative estimate of the prevalence of WNV antibody in medium-sized Wisconsin wild mammals.

We found similar serologic prevalence to WNV regardless of sex ($\chi^2 = 1.329$, degrees of freedom [df] = 1, p = 0.26), age ($\chi^2 = 1.31$, df = 1, p = 0.25), species ($\chi^2 = 3.64$, df = 2, p = 0.16), or month of collection after September (occurrence of WNV in avian species) ($\chi^2 = 1.42$, df = 1, p = 0.23). During our sampling period, the prevalence of WNV antibody was 27% (16/59) in coyotes, 20% (14/71) in opossums, and 19% (15/78) in raccoons. WNV antibody was found in 19 (18%) of 105 male animals compared with 26 (25%) of 103 female animals, and in 37 (21%) of 178 adults compared with 9 (30%) of 30 young of the year.

Mosquito transmission of WNV seems most likely in Wisconsin during August through September and less likely after frequent October frosts reduce the general mosquito population. In addition to mosquitoes, WNV may be transmitted by predation or scavenging (*6*,*10*). Previous studies (*3*,*6*), based on small sample sizes, reported relatively high WNV antibody prevalence rates for raccoons (>75%) and opossums (>60%). Our data indicate that the WNV antibody prevalence is similar for raccoons, opossums, and coyotes; however; food preferences differ in these 3 species (*11–13*). Raccoons are omnivorous, consuming mostly plant material, invertebrates, and small vertebrates; acorns and other plant materials are important fall food. Opossums are also omnivorous, consuming almost any available animal or plant material; their summer and fall diets consist primarily of invertebrates, small animals, and plant material. Coyotes are primarily predators on small vertebrates and scavengers on carcasses such as deer, livestock, and poultry. Because of the similarities in WNV antibody prevalence and differences in primary food choices, we suspect a common route of WNV transmission, most likely arthropodborne.

Table. Prevalence of West Nile virus (WNV) antibody in medium-sized wild mammals, southern Wisconsin, 2003–2004

		Virus antibody present	
Species	No. tested	Flavivirus, n (%)	WNV specific, n (%)
Raccoons	78	19 (24)	15 (19)
Opossums	71	27 (38)	14 (20)
Coyote	59	22 (37)	16 (27)
Red fox	7	1 (14)	1 (14)
Skunk	6	0	0
Feral cat	5	1 (20)	0
Badger	2	0	0
Total	228	70 (31)	46 (20)

A relatively high proportion of medium-sized mammals appear to have been infected with WNV. Whether these species play a role in maintenance and transmission of WNV needs to be determined. Whether raccoons, opossums, and coyotes can be indicators of WNV transmission or potential WNV reservoirs for subsequent transmission to avian, domestic animal, or human hosts is not known. Further research is needed to understand the role these species play in the epidemiology and epizootiology of WNV and the effect of the virus infection on specific populations of free-ranging mammals.

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Dr Docherty is diagnostic virologist emeritus for the US Department of the Interior, US Geologic Survey, NWHC. His primary duties consist of preparing manuscripts for publication and consulting on wildlife diagnostics and research.

References

1. Roehrig JT, Layton M, Smith P, Campbell GL, Nasci R, Lanciotti RS. The emergence of West Nile virus in North America: ecology, epidemiology, and surveillance. Curr Top Microbiol Immunol. 2002;267:223–40.
2. Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, et al. Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. Emerg Infect Dis. 2001;7:615–20.
3. Dietrich G, Montenieri JA, Panella NA, Langevin S, Lasater SE, Klenk K, et al. Serologic evidence of West Nile virus infection in free-ranging mammals, Slidell, LA, 2002. Vector Borne Zoonotic Dis. 2005;5:288–92.
4. Farajollahi A, Panella N, Carr P, Crans W, Burguess K, Komar N, et al. Serologic evidence of West Nile virus infection in black bears (*Ursus americanus*) from New Jersey. J Wildl Dis. 2003;39:894–6.
5. Santaella J, McLean R, Hall JS, Gill JS, Bowen RA, Hadow HH, et al. West Nile virus serosurveillance in Iowa white-tailed deer (1999–2003). Am J Trop Med Hyg. 2005;73:1038–42.
6. Root JJ, Hall J, McLean R, Marlenee N, Beaty B, Gansowski J, et al. Serologic evidence of exposure of wild mammals to flavivirus in the central and eastern United States. Am J Trop Med Hyg. 2005;72:622–30.
7. Blitvich BJ, Bowen RA, Marlenee NL, Hall RA, Bunning ML, Beaty BJ. Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile virus antibodies in domestic mammals. J Clin Microbiol. 2003;41:2676–9.

8. Joly DO, Ribic CA, Langenberg JA, Beheler K, Batha CA, Dhuey BJ, et al. Chronic wasting disease in free-ranging Wisconsin white-tailed deer. Emerg Infect Dis. 2003;9:599–601.
9. Beaty B, Calisher C, Shope R. Arboviruses. In: Lennette EH, Lennette DA, Lennette ET, editors. Diagnostic procedures for viral, rickettsial, and chlamydial infections: 7th ed. Washington: American Public Health Association;1995. p. 204–5.
10. Austgen LE, Bowen RA, Bunning ML, Davis BS, Mitchell CJ, Chang G-JJ. Experimental infection of cats and dogs with West Nile virus. Emerg Infect Dis. 2004;10:82–6.
11. Kaufmann JH. Raccoon and allies. In: Chapman JA, Feldhamer GA, editors. Wild mammals of North America: biology, management, and economics. Baltimore (MD): John Hopkins University Press; 1982. p. 573–4.
12. Gardner AL. Virginia opossum. In: Chapman JA, Feldhamer GA, editors. Wild mammals of North America: biology, management, and economics. Baltimore (MD): The John Hopkins University Press; 1982. p. 22–6.
13. Bekoff M. Coyote. In: Chapman JA, Feldhamer GA, editors. Wild mammals of North America: biology, management, and economics. Baltimore (MD): John Hopkins University Press; 1982. p. 452–3.

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Febrile Illness Associated with *Rickettsia conorii* Infection in Dogs from Sicily

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We report serologic and molecular evidence of acute, febrile illness associated with *Rickettsia conorii* in 3 male Yorkshire terriers from Sicily (Italy).

Rickettsia conorii, transmitted by *Ripicephalus sanguineus*, causes Mediterranean spotted fever (MSF) in humans in Mediterranean countries, Sub Saharan Africa and Asia (*1*). *Rickettsia* spp. seroprevalence in dogs is high (26%–60%) in disease-endemic regions, and proximity to seroreactive dogs is a risk factor for MSF in humans (*2*,*3*). Recent studies reported the detection of *Rickettsia* DNA in the blood of European dogs (*4*,*5*)**.** However, evidence that *R. conorii* infection causes illness in dogs is lacking (*2*,*3*,*6*). Illness has been associated with *R. conorii* natural infection in only 2 dogs since human MSF was described in 1932 (*6*). Moreover, the only clinical signs observed in experimentally infected dogs were pain, erythema, and edema at the injection site; and regional lymphadenopathy (*6*). We report infection with *R. conorii* ssp. *conorii* in 3 acutely ill, febrile Yorkshire terrier dogs, supported by PCR, DNA sequencing, and seroconversion.

The Study

Between May and September 2005, three unrelated intact male Yorkshire terriers with a mean age of 4.3 years from Catania, Sicily, were brought to a local veterinarian; the dogs had the following histories: anorexia and lethargy of 2 days' duration (dog 1); anorexia, lethargy, and intermittent lameness of a few days' duration (dog 2); and intermittent vomiting, anorexia, and lethargy of a few days' duration (dog 3). Despite living mostly indoors, all 3 dogs had a recent history of tick exposure. All dogs had received current vaccination histories and had no history of serious illness. Results of the physical examination and hematologic, biochemical, and serum electrophoresis abnormalities at the time of onset of clinical signs and after 1 month (dogs 2 and 3) and 2 months (dog 1) of follow-up are provided in Table 1. Treatments instituted for all 3 dogs at onset of illness are described in Table 1.

EDTA-blood and serum samples were obtained by the attending veterinarian at the time of clinical assessment (before treatment), then 1 week later and 1 month (dogs 2 and 3) or 2 months later (dog 1). DNA extraction was performed from whole blood samples (*5*,*7*). A quantitative PCR (qPCR) for detection of *Rickettsia* spp., *Anaplasma phagocytophilum*, *Ehrlichia canis,* and *Leishmania infantum* in DNA samples was performed by using a Light Cycler (Roche, Mannheim, Germany). PCR amplification was carried out with *Rickettsia* (Rr-prim3 5′-GAAACC GAAAGAGAATCTTCCGAT-3′ and Rr-prim4 5′-TCC TAGTGTAGAGGTGAAATTCTTA-3′ [*8*]), *E. canis*, *A. phagocytophilum* (fragment of 16S rRNA gene), and *L. infantum* LCSet primers and probes following manufacturer's instructions (TIB Molbiol, Centro Biotecnologie Avanzate, Genova, Italy) (*5*,*7*). Conventional *Babesia* genus PCR was performed (*9*). *Borrelia burgdorferi* sensu lato qPCR was performed by a commercial laboratory (www.scanelis.com). PCR results for all infectious agents listed above, with the exception of *Rickettsia,* were negative in all dogs.

PCRs for *Rickettsia* that use the outer membrane protein A (*ompA*) gene to amplify 632 bp (*10*) and 212 bp (107F 5′-GCTTTATTCACCACCTCAAC-3′ and 299R 5′-TRATCACCACCGTAAGTAAAT-3′) (*7*) amplicons were performed. For dog 1, a 632-bp amplicon was cloned by using the TOPO TA Cloning (Invitrogen, Carlsbad, CA, USA) and sequenced (GenBank accession nos. DQ518245) (*7*). For dogs 2 and 3, a 212-bp amplicon was subjected to direct sequencing (accession no. DQ518246, DQ518247) (*7*). PCR results are summarized in Table 1.

Consensus sequences were aligned [(BIOEDIT version 7.0 (ClustalW)] with known sequences in GenBank using the basic local alignment search tool (BLAST; available from http://www.ncbi.nlm.nih.gov/BLAST/). The sequence obtained from all 3 dogs was 100% homologous to a portion of the complete genome sequence corresponding to the *ompA* gene from *R. conorii* (Malish 7, accession no. AE008674).

Immunofluorescent assays to detect antibodies to *R. rickettsii*, *R. conorii*, *B. burgdorferi* sensu stricto, *E. canis*, *Babesia canis*, *A. phagocytophilum*, *L. infantum*, *Bartonella henselae,* and *B. vinsonii* ssp. *berkhoffi* antigens were performed (*3*,*7*). Results are presented in Table 2.

Conclusions

Clinicopathologic abnormalities detected in these dogs at initial examination, including acute onset of fever,

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Table 1. Clinical and laboratory data at the time of initial and follow-up examinations in 3 dogs with serologic and molecular evidence of natural *Rickettsia conorii* infection*

Date of evaluation, 2005	Physical examination abnormalities	Hematologic, biochemical, and serum electrophoresis abnormalities†	*Rickettsia* (16S and *ompA* gene) PCR
Dog 1			
May 31‡§	Fever (40.1ºC), tachycardia, mildly enlarged right popliteal lymph node, blepharitis, hunched posture, stiff gait	Left shift neutrophilia (segmented 11,700; bands: 468) and thrombocytopenia (112). ↑ ALT (112), hypoproteinemia (5.0) and hypoalbuminemia (40.4). ↑ α_2- (16.5) and β_1-globulins (13.9) ¶	Positive
Jun 8	No abnormalities	Mature neutrophilia (16,400); hyperglobulinemia (4.3); ↑ CRP (2.31); ↑ GGT (10.4); ↑ α_2- (18.5), γ-globulins (23.3); hypoalbuminemia (40.9)	Negative
Aug 8	No abnormalities	No abnormalities	Negative
Dog 2			
Sep 19‡§	Fever (41ºC), ptyalism, joint pain, lameness in right rear limb	Microcytic-hyperchromic anemia (MCHC 63.7; MCV 56; Hct 25.3); mature neutrophilia (11,680) ¶	Positive
Sep 28	No abnormalities	Hyperglobulinemia (4.3);↑ CRP (0.64); ↑ α_2- (17.5), and γ-globulin (24.6); hypoalbuminemia (41.1)	Negative
Oct 25	No abnormalities	No abnormalities	Negative
Dog 3			
Sep 17§#	Fever (41 ºC), abdominal pain, dehydration, peripheral lymphadenomegaly (popliteal and prescapular lymph nodes), conjunctivitis	Thrombocytopenia (69); hypoproteinemia (5.3); hypoalbuminemia (28.6); ↑ α_2- (14.8), β_1- (13.0), β_2- (15.6), γ-globulins (23.0) ¶	Positive
Sep 23‡	No abnormalities	Lymphocytosis (5,594); hyperglobulinemia (4.1); ↑ CRP (2.7); ↑ BUN (75); ↑ α_2-globulin (19.3); hypoalbuminemia (44.9)	Negative
Oct 25	No abnormalities	↑BUN (54)	Negative

*ALT, alanine aminotransferase; CRP, C-reactive protein; GGT, γ-glutamyl transferase; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; Hct, hematocrit; BUN, blood urea nitrogen; ↑, increase.
†Reference interval: Hct 38.6%–54.5%; MCV 61–72 fL; MCHC 34–38 g/dL; segmented neutrophils 3,800–8,800/μL; bands neutrophils 0–300/μL; lymphocytes 1,300–4,100/μL; platelets 160–440 × 10^3/μL); total protein 5.5–7.5 g/dL; globulins 2.6–4.0 g/dL; albumin 53%–65%; α_2-globulins 8.0%–14.0%; β_1-globulins 2.0%–5.0%; β_2-globulins 3.0%–9.0 %; γ-globulins 6.0%–15.0%); BUN 18–43 mg/dL; CRP 0.0–0.15 mg/dL; ALT 15–65 IU/L; GGT 2.0–8.0 IU/L.
‡Treatment with doxycycline (10 mg/kg/once a day by mouth/1 month) was started.
¶CRP was not measured
§Intravenous fluids were administered.
#Treatment with ceftriaxone (30 mg/kg/twice a day intravenously/for 5 days) was started.

lethargy, thrombocytopenia, anemia, mildly increased liver enzyme activities and hypoalbuminemia, were very similar to abnormalities associated with spotted fever group (SFG) rickettsioses in dogs and humans (*1*). In addition, *R. conorii* DNA was amplified in all dogs during the acute illness. Further evidence for *R. conorii* infection as a cause of the associated clinical signs was provided by the subsequent failure to detect DNA in dogs 1 and 2, 1 week after treatment with doxycycline and the rapid resolution of clinical signs 2 days after initiating doxycycline therapy. Clinical signs in dog 3 resolved in 4 days, while the dog was receiving ceftriaxone, which has no known anti-rickettsial efficacy (*1*). Spontaneous immune clearance of *R. conorii* likely accounted for the resolution of clinical signs in dog 3 (*6*).

The 4-fold increase in IgG antibody titers in dogs 2 and 3 supports seroconversion, which is consistent with an acute *R. conorii* infection (*11*). Additionally, the initially high IgM titer in dog 1 after the onset of illness compared with a much lower IgM titer after 65 days is also supportive of an acute infection and is consistent with observations of human serologic test results (*1*). IgM titers rise rapidly and then disappear by day 35 and 80 in dogs experimentally infected with *R. conorii* and *R. rickettsii*, respectively (*6*,*11*). However, high *R. rickettsii* IgM titers are detected in dogs that do not seroconvert, based upon IgG antibodies (*11*). Thus, the presence of IgM supports but does not prove acute SFG infection in dogs.

Coinfection with *A. phagocytophilum* or *B. burgdorferi* could have contributed to clinical signs observed in dog 1. This dog had a low serum *A. phagocytophilum* titer 7 days after initial examination and also seroconverted to *B. burgdorferi*. *A. phagocytophilum* causes an acute febrile illness in dogs and humans, similar to the findings described here (*12*). *B. burgdorferi* does not cause clinical signs in dogs until 60–150 days after experimental infection (*13*); therefore, despite seroconversion, the acute clinical signs in dog 1 were not likely to have been related to *B. burgdorferi* infection. Moreover, PCR amplification of DNA from organisms other than *R. conorii* was not found in any dog.

All dogs were intact, male, genetically unrelated Yorkshire terriers. Although an increased risk for Rocky Mountain spotted fever has not been reported in Yorkshire

Table 2. Reciprocal IFA titers for the 3 dogs with clinical and molecular evidence of natural *Rickettsia conorii* infection*

Dog no.	Date, 2005 (days after clinical signs)	*R. conorii*		*R. rickettsii*		*Anaplasma phagocytophilum*	*Borrelia burgdorferi*
		IgM	IgG	IgM	IgG	IgG	IgG
1	Jun 8 (7)	1:1,280	1:20,480	1:1,280	1:20,480	1:160	Neg
1	Aug 3 (65)	1:80	1:320	1:80	1:320	Neg	1:160
2	Sep 19 (0)	1:640	1:80	1:640	1:80	Neg	Neg
2	Sep 28 (9)	1:1,280	1:40,960	1:640	1:20,480	Neg	Neg
2	Oct 25 (37)	1:640	1:10,240	1:640	1:5,120	Neg	Neg
3	Sep 17 (0)	1:1,280	1:80	1:2,560	Neg	Neg	Neg
3	Sep 23 (6)	1:10,240	1:10,240	1:10,240	1:5,120	Neg	Neg
3	Oct 25 (39)	1:2,560	1:5,120	1:5,120	1:5,120	Neg	Neg

*IFA, immunofluorescence assay; Ig, immunoglobulin; Neg, negative.
†All dogs were seronegative for *L. infantum, E. canis, B. canis, B. henselae,* and *B. vinsonii* ssp. berkhoffi at all time points

terriers, purebred dogs infected with *R. rickettsii* appear to be more prone to clinical illness (*14*). Notably, this breed seems to be at increased risk for *Babesia canis* infection (*15*). Male dogs and men may be at increased risk for infection and may develop more severe illness with *R. rickettsii* and *R. conorii* (*1*,*14*), and male dogs are more likely to be *R. conorii* seroreactive (*3*). It has been suggested that more severe illness may develop in English springer spaniels with suspected phosphofructokinase deficiency and persons with glucose 6-phosphate dehydrogenase deficiency when infected with *R. rickettsii* and *R. conorii* (*1*,*14*). Although inherited immunodeficiencies have not been reported in Yorkshire terriers, and all dogs were previously healthy, an inherited metabolic or immunologic defect cannot be ruled out because specific testing was not performed.

Although a metabolic or immunologic defect may be necessary for illness to develop in dogs of various breeds after *R. conorii* infection, other potential explanations can be made for the discrepancy between high *R. conorii* seroprevalence rates among healthy dogs and lack of reports of clinical illness. The high *R. conorii* seroprevalence in healthy dog populations suggests that exposure to SFG rickettsiae is common. However, the acute, nonspecific, and potentially self-limiting nature of *R. conorii* infection, combined with a low index of suspicion by regional veterinarians and a historical lack of specific diagnostic techniques, may have precluded the prior association of clinical signs with *R. conorii* infection in dogs. Further evidence should be gathered regarding the extent to which *R. conorii* causes clinical disease in dogs.

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References

1. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev. 2005;18:719–56.
2. Segura-Porta F, Diestre-Ortin G, Ortuno-Romero A, Sanfeliu-Sala I, Font-Creus B, Munoz-Espin T, et al. Prevalence of antibodies to spotted fever group rickettsiae in human beings and dogs from an endemic area of Mediterranean spotted fever in Catalonia, Spain. Eur J Epidemiol. 1998;14:395–8.
3. Solano-Gallego LJ, Osso M, Hegarty B, Breitschwerdt E. A serological study of exposure to arthropod-borne pathogens in dogs from northeastern Spain. Vet Res. 2006;37:231–44.
4. Estrada-Pena A, Venzal Bianchi J. Efficacy of four anti-tick chemicals to break the transmission of *Rickettsia conorii* to dogs. In: Abstracts of the Fourth International Conference on Rickettsiae and Rickettsial Diseases, Logroño, Spain; 2005 Jun 18–21. P-100.
5. Solano-Gallego L, Razia L, Trotta T, Furlanello T, Caldin M. Molecular survey of *Ehrlichia canis, Anaplasma phagocytophilum* and *Rickettsia* spp. from blood of dogs living in Italy. In: Abstracts of the Fourth International Conference on *Rickettsiae* and Rickettsial Diseases, Logroño, Spain; 2005 Jun 18–21. P-107.
6. Kelly PJ, Matthewman LA, Mason PR, Courtney S, Katsande C, Rukwava J. Experimental infection of dogs with a Zimbabwean strain of *Rickettsia conorii.* J Trop Med Hyg. 1992;95:322–6.
7. Kidd LB. Evaluation of a PCR assay for detection of spotted fever group *Rickettsia* in dog blood. North Carolina State University; 2006 [PhD dssertation].
8. Breitschwerdt EB, Papich MG, Hegarty BC, Gilger B, Hancock SI, Davidson MG. Efficacy of doxycycline, azithromycin, or trovafloxacin for treatment of experimental Rocky Mountain spotted fever in dogs. Antimicrob Agents Chemother. 1999;43:813–21.
9. Carret C, Walas F, Carcy B, Grande N, Precigout E, Moubri K, et al. *Babesia canis canis, Babesia canis vogeli, Babesia canis rossi:* differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. J Eukaryot Microbiol. 1999;46:298–303.
10. Roux V, Fournier PE, Raoult D. Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. J Clin Microbiol. 1996;34:2058–65.
11. Breitschwerdt EB, Levy MG, Davidson MG, Walker DH, Burgdorfer W, Curtis BC, et al. Kinetics of IgM and IgG responses to experimental and naturally acquired *Rickettsia rickettsii* infection in dogs. Am J Vet Res. 1990;51:1312–6.
12. Neer TM, Breitschwerdt EB, Greene RT, Lappin MR. Consensus statement on ehrlichial disease of small animals from the infectious disease study group of the ACVIM. American College of Veterinary Internal Medicine. J Vet Intern Med. 2002;16:309–15.

13. Greene CE, Straubinger RK. Borreliosis. In: Green C, ed. Infectious diseases of the dog and cat. 3rd ed. Philadelphia: Saunders, Elsevier; 2006. p. 417–34.
14. Greene CE, Breitschwerdt EB. Rocky Mountain spotted fever, murine typhuslike disease, rickettsialpox, typhus, and Q fever. In: Greene C, editor. Infectious diseases of the dog and cat. 3rd ed. Philadelphia: Saunders, Elsevier; 2006. p. 232–45.
15. Martinod S, Laurent N, Moreau Y. Resistance and immunity of dogs against *Babesia canis* in an endemic area. Vet Parasitol. 1986;19:245–54.

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Genotyping of *Chlamydophila psittaci* in Human Samples

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Chlamydophila (formerly *Chlamydia*) *psittaci* genotypes A, B, C, and a new genotype most similar to the 6BC type strain were found in 10 humans with psittacosis by outer membrane protein A gene sequencing. Genotypes B (n = 3) and C (n = 1) are endemic in nonpsittacine European birds. These birds may represent an important part of the zoonotic reservoir.

Psittacosis is a zoonosis caused by infection with *Chlamydophila* (formerly *Chlamydia*) *psittaci*, an obligate intracellular bacterium. *C. psittaci* is divided into 8 serovars (A–F, M56, and WC) and at least 9 genotypes. Sequence analysis of the outer membrane protein A (*ompA*) gene is the most accurate method for identifying all known genotypes (*1*). All genotypes are associated with specific bird groups from which they are predominantly isolated (*2*,*3*). The prevalence of different genotypes of *C. psittaci* that cause infection in humans is unknown. In this study, we genotyped all *C. psittaci* PCR-positive human samples available in our laboratory.

The Study

Ten human samples positive for *C. psittaci* DNA in our previously described real-time PCR assay were characterized by *ompA* gene sequencing (*4*). These samples were collected from 2002 through 2005 and included 4 sputum, 4 bronchoalveolar lavage, 1 throat swab, and 1 serum. All samples were obtained from symptomatic psittacosis patients admitted to hospitals in the Netherlands. All patients had pneumonia, and 6 required treatment in an intensive care unit. The DNA of 1 outbreak strain, which infected as many as 10 people, was tested only once in this study. One of the samples was obtained from a patient who has been previously described (*5*)

DNA purification was performed by using the guanidinium thiocyanate–silica extraction procedure (*6*). Genotyping was performed essentially as previously described (*7*). Briefly, a part of the *ompA* gene was amplified with primers CPsittGenoFor (5′-GCT ACG GGT TCC GCT CT-3′) and CPsittGenoRev (5′-TTT GTT GAT YTG AAT CGA AGC-3′). These primers are located in the conserved regions of the *ompA* gene that contains the 4 variable domains (VDs). On the basis of published *ompA* sequence of the *C. psittaci* 6BC type strain (GenBank accession no. X56980), the resulting amplicon should have a size of 1,041 bp. PCR products were analyzed by agarose gel electrophoresis, and the expected 1,041-bp amplicon was excised from the gel. DNA was extracted from the gel and reamplified for 20 cycles, and amplicons were analyzed for size by agarose gel electrophoresis. *C. psittaci* ORNI (genotype A) strain and a *C. abortus* strain were used as positive controls. Calf thymus DNA was used as a negative control.

If the *ompA* gene could not be amplified with this procedure, a nested PCR with primers CPsittFinner (5′-CGC TCT CTC CTT ACA AGC C-3′) and CPsittRinner (5′-GAT CTG AAT CGA AGC AAT TTG-3′) was used. The amplified product (n = 8) or the nested PCR product (n = 2) were subjected to sequence analysis (BigDye Terminator, version 1.1, cycle sequencing kit, Applied Biosystems, Foster City, CA, USA). Overlapping sequences were obtained with 6 sequencing primers: CPsittGenoFor and CPsittGenoRev, inner primers CPsittFinner and CPsittRinner, and primers CPsittHFor (5′-TCT TGG AGC GTR GGT GC-3′) and CPsittHRev (5′-GCA CCY ACG CTC CAA GA-3′).

The resulting sequences were aligned, and a similarity index based on the translation of the 984-bp gene fragment was calculated. Similarity (1 – distance) was calculated by using the pairwise distance method generated by MEGA3 (*8*). Reference *ompA* genotype sequences A–F and the *ompA* sequence of the *C. psittaci* 6BC type strain available in GenBank (accession nos. AY762608–AY762612, X56980, and AF269261) were included in this analysis (*1*,*9*).

All 10 isolates could be genotyped. The *ompA* sequence of 5 isolates was identical to the sequence of reference genotype A, 3 isolates were identical to genotype B, and the *ompA* sequence of 1 isolate was identical to genotype C. One isolate had a novel *ompA* sequence type that was 99.4% similar to the genotype A reference but more similar to the *C. psittaci* 6BC type strain (99.7%). Two nonsynonymous mutations were present in this sequence compared with reference genotype A. A substitution of thymine for adenine in VD 1 resulted in Ser instead of Thr at amino acid position 92 of the *ompA* amino acid sequence, which is identical to that found in genotype C. A substitution of cytosine for guanine, also located in VD 1, resulted in Gln instead of Glu at amino acid position 117, as found in genotype B and strain 6BC (numbering accord-

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ing to the *ompA* amino acid sequence of the *C. psittaci* 6BC strain, GenBank accession no. X56980). We designated this new variant *C. psittaci* 05/02 and deposited the sequence in GenBank (accession no DQ324426). Two genotype B strains, 3 genotype A strains, and the novel genotype 05/02 strain were obtained from patients admitted to an intensive care unit.

Conclusions

To our knowledge, ours is the first report of a series of genotyped *C. psittaci* strains isolated from symptomatic, hospitalized patients. These 10 samples reflect approximately one third of all cases reported each year in the Netherlands (*10*). From the genotypes that we identified, we may infer the zoonotic reservoirs of *C. psittaci* in the Netherlands. The different genotypes of *C. psittaci* are associated, although not exclusively, with different birds from which they are mostly isolated. Genotype A is mainly found in psittacine birds and was the most prevalent genotype in our samples (*1*,*3*). *C. psittaci* 05/02 was most related to *C. psittaci* 6BC and the reference genotype A (strain VS1). Both reference strains have been classified as serovar A strains. On the basis of 2 restriction fragment length polymorphism patterns, Sayada et al. suggested that serovar A should be divided into 2 genogroups (*2*). Our isolate 05/02 is a new *ompA* gene sequence variant within this probably heterogeneous group.

Genotype B has been isolated mainly from feral pigeons and several other bird species; this genotype is considered endemic in European nonpsittacine birds (*3*,*11*). Genotype C has been isolated mainly from ducks, and we detected this genotype in 1 of our human samples. We did not find genotype D, which is prevalent among poultry, especially turkeys, or genotypes E and F. These last 2 genotypes are rare and found occasionally in birds (*1*,*11*). Imported psittacine birds, which carry mainly genotype A, have been proposed as the major source for human psittacosis infections (*12*). In our study, 4 of 10 isolates were genotypes B and C, which are rarely found in psittacine birds. This finding suggests that nonpsittacine birds may represent an underestimated source for human psittacosis cases.

We detected isolates of genotypes A, B, C, and a new genotype similar to the *C. psittaci* 6BC strain in a series of 10 *C. psittaci*–positive samples. Genotypes B and C are endemic in European nonpsittacine birds, which may represent an important part of the zoonotic reservoir for human psittacosis cases.

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References

1. Geens T, Desplanques A, van Loock M, Bonner BM, Kaleta EF, Magnino S, et al. Sequencing of the *Chlamydophila psittaci ompA* gene reveals a new genotype, E/B, and the need for a rapid discriminatory genotyping method. J Clin Microbiol. 2005;43:2456–61.
2. Sayada C, Andersen AA, Storey C, Milon A, Eb F, Hashimoto N, et al. Usefulness of *omp1* restriction mapping for avian *Chlamydia psittaci* isolate differentiation. Res Microbiol. 1995;146:155–65.
3. Vanrompay D, Andersen AA, Ducatelle R, Haesebrouck F. Serotyping of European isolates of *Chlamydia psittaci* from poultry and other birds. J Clin Microbiol. 1993;31:134–7.
4. Heddema ER, Beld MG, de Wever B, Langerak AA, Pannekoek Y, Duim B. Development of an internally controlled real-time PCR assay for detection of *Chlamydophila psittaci* in the LightCycler 2.0 system. Clin Microbiol Infect. 2006;12:571–5.
5. Heddema ER, Kraan MC, Buys-Bergen HE, Smith HE, Wertheim-Van Dillen PM. A woman with a lobar infiltrate due to psittacosis detected by polymerase chain reaction. Scand J Infect Dis. 2003;35:422–4.
6. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-Van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990;28:495–503.
7. Heddema ER. ter Sluis S, Buijs JA, Vandenbroucke-Grauls CM, van Wijnen JH, Visser CE. Prevalence of *Chlamydophila psittaci* in fecal droppings from feral pigeons in Amsterdam, The Netherlands. Appl Environ Microbiol. 2006;72:4423–5.
8. Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 2004;5:150–63.
9. Bush RM, Everett KD. Molecular evolution of the *Chlamydiaceae*. Int J Syst Evol Microbiol. 2001;51:203–20.
10. National Institute of Public Health and the Environment (RIVM). Notified cases of infectious diseases in the Netherlands. Dutch Infectious Diseases Bulletin. 2004;15:30.
11. Vanrompay D, Butaye P, Sayada C, Ducatelle R, Haesebrouck F. Characterization of avian *Chlamydia psittaci* strains using *omp1* restriction mapping and serovar-specific monoclonal antibodies. Res Microbiol. 1997;148:327–33.
12. Wreghitt TG, Taylor CE. Incidence of respiratory tract chlamydial infections and importation of psittacine birds. Lancet. 1988;1:582.

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Community-associated Methicillin-resistant *Staphylococcus aureus* and Healthcare Risk Factors

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To determine frequency of methicillin-resistant *Staphylococcus aureus* infections caused by strains typically associated with community-acquired infections (USA300) among persons with healthcare-related risk factors (HRFs), we evaluated surveillance data. Of patients with HRFs, 18%–28% had a "community-associated" strain, primarily USA300; of patients without HRFs, 26% had a "healthcare-associated" strain, typically USA100.

In the United States, initial reports of methicillin-resistant *Staphylococcus aureus* (MRSA) infections among injection drug users in Detroit in 1981 were followed by reports of MRSA associated with the deaths of 4 children in Minnesota and North Dakota in 1997 (*1*). For the next few years, public health personnel in several states investigated outbreaks of MRSA infections of skin and soft tissue among diverse populations who typically had little or no previous contact with the healthcare system, such as Native Americans (*2*), sports teams (*3*), prison inmates (*4*), and child-care facility attendees (*5*). These outbreaks were initially associated with a novel MRSA strain known as MW2, or pulsed-field gel electrophoresis (PFGE) type USA400, but were soon replaced by a strain of MRSA belonging to PFGE type USA300 (*6*). Through 2002, the clinical appearance of cases and the microbiologic characteristics of USA300 and USA400 differed substantially from those associated with strains of MRSA acquired in healthcare settings (*7*). Increasingly, MRSA strains of community origin are causing healthcare-associated disease (*8*,*9*). We evaluated surveillance data from a multisite project to determine the frequency with which infections among patients with healthcare-related risk factors (HRFs) were caused by USA300 or other strains of community origin.

The Study

Active, population-based surveillance for invasive MRSA infections is ongoing in 9 US states (California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New York, Oregon, and Tennessee) through the Active Bacterial Core Surveillance system in the Emerging Infections Program at the Centers for Disease Control and Prevention (CDC). Personnel in each state actively collect laboratory reports of positive MRSA cultures from normally sterile sites (e.g., blood; cerebrospinal, joint, or pleural fluid) of residents in their catchment areas to identify cases. In 2005, the estimated combined population under surveillance was 16.3 million, according to data from the US Bureau of the Census. To report a case, personnel must link a laboratory report to the patient's medical record. During record reviews, personnel abstract information about the following HRFs: culture obtained >48 hours after admission; presence of an invasive device (e.g., vascular catheter, G-tube); and history of MRSA infection or colonization, surgery, hospitalization, dialysis, or residence in a long-term care facility in the 12 months preceding the culture. Case-patients may have >1 HRF. For this analysis, we used information from the record review to classify cases into 3 mutually exclusive groups: 1) case-patients with classic healthcare-associated infections (HA) whose culture was obtained >48 hours after admission

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Table 1. Selected characteristics among case-patients with invasive MRSA, by healthcare-related risk factors, Active Bacterial Core Surveillance, January 2004–February 2006*

Characteristic	With HRFs, no. (%)		Without HRFs, no. (%)
	Healthcare-associated, n = 2,535	Healthcare-associated, community onset, n = 5,353	Community-associated,† n = 1,259
Median age, y	62‡	62‡	46
Pneumonia	413 (16.3)	685 (12.8)‡	190 (15.1)
Endocarditis	72 (2.8)‡	345 (6.4)‡	158 (12.6)
Died	687 (27.1)‡	845 (15.8)‡	131 (10.4)

*MRSA, methicillin-resistant *Staphylococcus aureus;* HRFs, healthcare-related risk factors.
†Patients with community-associated infections were those who did not have HRFs; these patients were used as reference.
‡$p<0.05$ for χ^2 test for categorical variables; Wilcoxon rank sum for age.

with or without other HRFs; 2) case-patients with HRFs but with community onset (i.e., whose cultures were obtained ≤48 hours after admission) (HACO); and 3) case-patients with community-associated (CA) infections without HRFs, according to medical record review.

A subset of isolates from case-patients was collected from laboratories that voluntarily submitted them for microbiologic characterization. Of the isolates received at CDC by October 2005, a sample of 100 was selected for testing as follows. First, isolates were stratified by Emerging Infections Program site; none were available from Maryland. Second, all isolates from tissues other than blood were selected from each Emerging Infections Program site. To ensure 12–13 isolates per site, we selected blood isolates from case-patients classified as CA and obtained the remainder from samples from HA and HACO case-patients. Isolates were tested by PFGE; patterns were analyzed by using BioNumerics (Applied Maths, Austin, TX, USA). Isolates were grouped into PFGE types using Dice coefficients and 80% relatedness (*10*). We considered isolates with PFGE types USA300, 400, or 1000 to be of community origin and those with types USA100, 200, and 500 to be of healthcare origin as previously described (*10*).

Statistical analysis consisted of comparisons of proportions between CA and HA and between CA and HACO cases using χ^2 pairwise comparisons. Differences in median age were tested by using Wilcoxon rank sum test.

Of 9,147 cases of invasive MRSA infection investigated from January 2004 through February 2006, 2,535 (28%) were HA, 5,353 (59%) were HACO, and 1,259 (14%) were CA. The median age of case-patients with HA and HACO was significantly higher than that of case-patients with CA (Table 1). CA case-patients were 1) more likely to have pneumonia than HACO but not HA case-patients; 2) more likely to have endocarditis than either HA or HACO case-patients; and 3) less likely to die during this hospital stay than were HA or HACO case-patients.

Of the 100 isolates selected for initial testing, 29 were from HA case-patients, 44 were from HACO case-patients (including 1 isolate of a unique PFGE type), and 27 were from CA case-patients (including 1 isolate that could not be typed) (Table 2). Of the HA isolates, 8 (28%) were USA300. Of the HACO isolates, 6 (14%) were USA300, 1 (2%) was USA400, and 1 (2%) was USA1000. Thus, 18%–28% of isolates in patients with HRFs (HA and HACO) had PFGE patterns typical of community strains. Of the 27 isolates from CA case-patients, 5 (19%) were USA100 and 2 (7%) were USA500; thus, 7 (26%) of isolates among CA case-patients were strains typically considered to be of healthcare origin.

Conclusions

MRSA strains such as USA300, which were initially a cause of MRSA infections in the community, have migrated into healthcare settings. The results from this multisite project are consistent with observations from individual facilities, where USA300 isolates caused illness in patients whose infection was healthcare associated (*11*,*12*).

Table 2. MRSA isolates from invasive sites by healthcare-related risk factors and PFGE type, Active Bacterial Core Surveillance, January 2004–February 2006*

PFGE type	Healthcare associated, no. (%)	Healthcare associated, community onset, no. (%)	Community associated,† no. (%)	Total, no. (%)
USA100	20 (69)	30 (68)	5 (19)	55 (55)
USA200	1 (3)	0	0	1 (1)
USA300	8 (28)	6 (14)	18 (67)	32 (32)
USA400	0	1 (2)	0	1 (1)
USA500	0	5 (11)	2 (7)	7 (7)
USA1000	0	1 (2)	1 (4)	2 (2)
Unique type	0	1 (2)	0	1 (1)
Not typeable	0	0	1 (4)	1 (1)
Total	29 (100)	44 (100)	27 (100)	100 (100)

*MRSA, methicillin-resistant *Staphylococcus aureus;* PFGE, pulsed-field gel electrophoresis.
†Patients with community-associated infections were those who did not have healthcare-related risk factors.

Although age and frequency of endocarditis still differed between case-patients with HRFs (HA and HACO) and those without HRFs (CA), PFGE testing indicated that 18%–28% of patients with HRFs were infected with a "community-associated" strain of MRSA, primarily USA300. Furthermore, 26% of patients without HRFs had a "healthcare-associated" strain, typically USA100. Thus, the distinction between healthcare- and community-associated MRSA is rapidly blurring.

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Dr Klevens is a medical epidemiologist at CDC. She is the CDC principal investigator in a multistate project that measures methicillin-resistant *Staphylococcus aureus* in the population, and she provides epidemiologic support to the National Healthcare Safety Network.

References

1. Centers for Disease Control and Prevention. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*—Minnesota and North Dakota, 1997–1999. JAMA. 1999;282:1123–5.
2. Baggett HC, Hennessy TW, Rudolph K, Bruden D, Reasonover A, Parkinson A, et al. Community-onset methicillin-resistant *Staphylococcus aureus* associated with antibiotic use and the cytotoxin Panton-Valentine leukocidin during a furunculosis outbreak in rural Alaska. J Infect Dis. 2004;189:1565–73.
3. Centers for Disease Control and Prevention. Methicillin-resistant *Staphylococcus aureus* infections among competitive sports participants—Colorado, Indiana, Pennsylvania, and Los Angeles County, 2000–2003. MMWR Morb Mortal Wkly Rep. 2003;52:793–5.
4. Centers for Disease Control and Prevention. Public health dispatch: outbreaks of community-associated methicillin-resistant *Staphylococcus aureus* skin infections—Los Angeles County, California, 2002–2003. JAMA. 2003;289:1377.
5. Adcock PM, Pastor P, Medley F, Patterson JE, Murphy TV. Methicillin-resistant *Staphylococcus aureus* in two child care centers. J Infect Dis. 1998;178:577–80.
6. Tenover FC, McDougal LK, Goering RV, Killgore G, Projan SJ, Patel JB, et al. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J Clin Microbiol. 2006;44:108–18.
7. Fridkin SK, Hageman JC, Morrison M, Sanza LT, Como-Sabetti K, Jernigan JA, et al. Methicillin-resistant *Staphylococcus aureus* disease in three communities. N Engl J Med. 2005;352:1436–44. Erratum in: N Engl J Med. 2005;352:2362.
8. Healy CM, Hulten KG, Palazzi DL, Campbell JR, Baker CJ. Emergence of new strains of methicillin-resistant *Staphylococcus aureus* in a neonatal intensive care unit. Clin Infect Dis. 2004;39:1460–6.
9. Saiman L, O'Keefe M, Graham PL III, Wu F, Said-Salim B, Kreiswirth B, et al. Hospital transmission of community-acquired methicillin-resistant *Staphylococcus aureus* among postpartum women. Clin Infect Dis. 2003;37:1313–9.
10. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol. 2003;41:5113–20.
11. Seybold U, Kourbatova EV, Johnson JG, Halvosa SJ, Wang YF, King MD, et al. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. Clin Infect Dis. 2006;42:647-56.
12. Huang H, Flynn NM, King JH, Monchaud C, Morita M, Cohen SH. Comparisons of community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) and hospital-associated MSRA infections in Sacramento, California. J Clin Microbiol. 2006; 44:2423-7.

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Chikungunya Disease Outbreak, Reunion Island

To the Editor: A serious outbreak of chikungunya disease recently occurred on Reunion Island (population ≈770,000) (*1*). Between March 1, 2005, and April 30, 2006, ≈255,000 cases were reported in this French territory in the Indian Ocean. Most cases occurred after mid-December 2005, with a maximum of 45,000 cases during the week of January 29 to February 4, 2006 (*2*). Surveillance figures were confirmed by a serosurvey that found a prevalence of 18% of recent infection markers in pregnant women in March 2006.

Chikungunya is a self-limiting febrile viral disease characterized by arthralgia or arthritis. Symptoms may last for several months, but recovery was, until now, considered universal (*3*). However, in January 2006, the health authorities on this island started receiving death certificates mentioning chikungunya as a cause of death, either direct or indirect. By the end of April, 213 death certificates with this finding had been received. To assess the affect of chikungunya disease, we compared the crude death rate (CDR) observed during the outbreak period with an expected death rate computed from the 2002–2004 historical data.

The study included the period January 1, 2005, through April 30, 2006. The expected number of deaths (all causes) for 2005 and 2006 was the number of deaths by sex and age observed during 2002–2004 modified by an estimation of the population size for 2005–2006. The details of this method, which was used during the heat wave in France in 2003, have been reported (*4*). The number of deaths in Reunion was obtained daily from 13 of 24 computerized registry offices throughout the island and represented 87% of the deaths on the island.

During 2005, the monthly CDR remained within expected range of statistical variation. From January through April 2006, respectively, monthly CDRs were 7.1%, 34.4%, 25.2%, and 8.3% higher than expected rates ($p<0.01$ for February and March). This corresponded to 226 excess deaths reported by the 13 offices participating in the study and 260 excess deaths when data were extrapolated to the entire population of the island (an increase of 18.4%) (Figure). The 260 excess deaths is a crude figure that includes potentially all causes of death. This figure leads to a rough estimate of the case-fatality rate for chikungunya disease of ≈1/1,000 cases. Excess deaths were observed mainly in persons ≥75 years of age.

CDRs began to exceed the expected range during the last week of January 2006 and remained elevated until the end of the study period. This situation closely matched the kinetics of the epidemic curve of chikungunya disease. CDR is a stable variable in time for a defined population. Only a massive phenomenon can have an effect on it, and no other abnormal health event affected the island at this time. Thus, the outbreak of chikungunya disease was likely responsible for most of the excess deaths observed in Reunion during the first 4 months of 2006.

Deaths associated with chikungunya disease have been rarely reported. This outbreak in Reunion is the first with such a high incidence in a setting where real-time death reporting is a standard procedure. In such settings, CDR monitoring should be considered syndromic surveillance and should be implemented when an abnormal health phenomenon affects large populations.

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References

1. Enserink M. Infectious diseases. Massive outbreak draws fresh attention to little-known virus. Science. 2006;311:1085.
2. Paganin F, Borgherini G, Staikowsky F, Arvin-Berod C, Poubeau P. Chikungunya on Reunion Island: chronicle of an epidemic foretold. Presse Med. 2006;35:641–6.
3. Mackenzie JS, Chua KB, Daniels PW, Eaton BT, Field HE, Hall RA, et al. Emerging viral diseases of Southeast Asia and the Western Pacific. Emerg Infect Dis. 2001;7(3 Suppl):497–504.

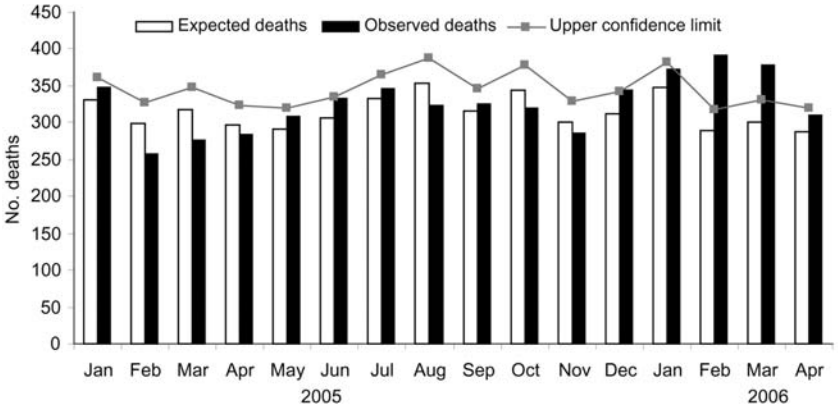

Figure. Expected and observed number of deaths reported by 13 computerized registry offices in Reunion Island, France, January 2005–April 2006.

4. Pirard P, Vandentorren S, Pascal M, Laaidi K, Le Tertre A, Cassadou S, et al. Summary of the mortality impact assessment of the 2003 heat wave in France. Euro Surveill. 2005;10:153–6.

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Assay to Detect H5N1 Oseltamivir Resistance

To the Editor: Oseltamivir is a neuraminidase inhibitor approved for treatment and prevention of influenza virus infection. Oseltamivir resistance caused by a single amino acid substitution from histidine (H) to tyrosine (Y) at position 274 of the neuraminidase active site has been reported in persons infected both experimentally and naturally with influenza A virus subtype H5N1 (*1*,*2*). Evidence suggests that using lower doses of oseltamivir or shorter-than-recommended treatment periods can contribute to emergence of viral resistance (*1*,*3*) Currently, oseltamivir is being used in several countries that may be affected by epidemics of H5N1. Therefore, monitoring for oseltamivir-resistant strains of H5N1 during oseltamivir administration is essential for outbreak management and prevention.

Although real-time PCR or pyrosequencing is more rapid than high-throughput assays for mutation detection (*4*,*5*), the conventional PCR technique can be applied to detect drug-resistant mutation (*6*) in areas lacking real-time PCR or pyrosequencing capabilities. Therefore, to discriminate between oseltamivir-sensitive and oseltamivir-resistant strains, we developed a simple method, based on PCR, which takes advantage of the H274Y substitution. The forward primer was designed from the conserved region common to both wild-type and mutant strains; the reverse primers were designed specifically for wild-type and mutant strains, respectively, derived from the 3′ terminal base of each primer. The primers consisted of a forward primer N1f (nt 517-534: 5′-GGGGCTGTGGCTGTATTG-3′) and reverse primer H274r (nt 759-784: 5′-GGATAACAGGAGCAYTCCTCATAGTG -3′) for wild-type strain detection or Y274r (nt 759-784: 5′-GGATAACAGGAGCAYTCCTCATAGTA-3′) for mutant strain detection. (Note: Underlined letters represent differences in nucleotides between plus vs. minus primers.) Both strains yielded products of ≈267 bp; hence, the assay consisted of 2 separate reactions for detecting wild-type and mutant strains, respectively.

For each reaction, 1.0 μL cDNA was combined with a reaction mixture that contained 10 μL 2.5× MasterMix (Eppendorf, Hamburg, Germany), forward and reverse primers at a final concentration of 0.15 μM, and nuclease-free water to a final volume of 20 μL. Thermocycling conditions comprised initial denaturation at 94°C for 3 min and 35 cycles of amplification including denaturation (94°C, 30 s), annealing (65°C, 50 s), extension (72°C, 45 s), and final extension (72°C, 7 min). Subsequently, 10 μL of the amplified products was analyzed by using 2% agarose gel electrophoresis.

To optimize the assay, we performed PCR-based H274Y mutagenesis of the N1 fragment of the H5N1 virus (primers on request). The resulting mutagenic and wild-type products were cloned into the pGEM-T Easy Vectors (Promega, Madison, WI, USA), confirmed by direct sequencing, and then used as positive controls. Preliminary results showed that the wild-type primer was specific for the oseltamivir-sensitive strain, whereas the mutant primer can be used to detect the oseltamivir-resistant strain exclusively because no significant cross-amplification had been observed.

To establish sensitivity, serial 10-fold dilutions of the standard N1 plasmids (wild-type and mutant) ranging from 10^9 to 10^1 copies/μL were used as a template. The threshold concentrations for detection of wild-types and mutants were 10^3 copies/μL. To provide semiquantitative data to detect subpopulations of the resistant variants, the 2 control plasmids were mixed at wild-type:variant ratios of 10^8:10^2, 10^7:10^3, 10^6:10^4, 10^5:10^5, 10^4:10^6, 10^3:10^7, and 10^2:10^8. The result showed that the density of the expected bands depended on the amount of DNA templates (Figure B). However, the mixing experiments indicated that the predominant mixtures of wild-type:resistant variant were 80:20, which is the lowest ratio of resistant variants that the assay can reliably detect (data not shown). To assess specificity, human DNA and viral cDNA extracted from other subtypes of influenza A virus (N2–N9) were subjected to this assay. No cross-reaction occurred with human DNA or other subtypes of influenza A virus.

We further validated the assay by testing 3 specimens from hosts treated with oseltamivir and 17 specimens from untreated hosts; infection with H5N1 was detected by using multiplex real-time PCR (*7*). The specimens from oseltamivir recipients were isolated from a Vietnamese patient on the third day after he received a prophylactic dose (*1*) and from 2 tigers (CU-T7 and KU-11) (*8*). The specimens from untreated hosts were isolated from the plasma of an H5N1-infected human (*9*) and from virus-containing allantoic fluid isolated from infected chickens, ducks,

Figure. A) Representative result from conventional PCR that used H274r primer for oseltamivir-sensitive and Y274r primer for oseltamivir-resistant detection in samples isolated from human plasma (P), tiger (T), and Vietnamese patient (V). Plasmids containing N1 fragments obtained from PCR-based mutagenesis for wild-type H274 (Wt) and mutant Y274 (Mt) were used as positive controls in each reaction. (N, no template control; M, 100-bp molecular marker.) B) Semiquantitative data on the ability of the assay to detect subpopulations of the resistant variants. The 2 control plasmids were mixed at wild-type:variant ratios of 10^8:10^2 (lanes 1 and 8), 10^7:10^3 (lanes 2 and 9), 10^6:10^4 (lanes 3 and 10), 10^5:10^5 (lanes 4 and 11), 10^4:10^6 (lanes 5 and 12), 10^3:10^7 (lanes 6 and 13), and 10^2:10^8 (lanes 7 and 14).

and cats (n = 16) during a 2005 outbreak in Thailand. The specimen isolated from the Vietnamese patient yielded detectable bands after amplification by wild-type and mutant primer sets, which indicates that this specimen contains mixed populations of wild-type and resistant strains (Figure, A). The result was confirmed by cloning the amplicon of the Vietnamese strain into the pGEM-T EASY vector (Promega). Ten colonies were randomly picked and sequenced; 9 clones were mutant, and 1 was wild type. The other specimens produced a visible positive band only on amplification using the wild-type primer set, which indicates that samples containing these strains were oseltamivir sensitive.

The assay described here provides an accurate, cost-effective, and highly efficient approach to detecting oseltamivir-sensitive and oseltamivir-resistant H5N1 strains; it is based on conventional PCR and takes advantage of the H274Y substitution within the neuraminidase gene. This simple technique can be applied in all laboratories that lack more advanced and expensive instruments; thus, it provides a valuable tool for managing and preventing influenza A H5N1 outbreaks. Concerning the spread of mutant viruses, however, the fitness of the viruses needs further investigation.

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References

1. Le QM, Kiso M, Someya K, Sakai YT, Nguyen TH, Nguyen KH, et al. Avian flu: isolation of drug-resistant H5N1 virus. Nature. 2005;437:1108.
2. de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, Nguyen VC, et al. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med. 2005;353:2667–72.
3. Ward P, Small I, Smith J, Suter P, Dutkowski R. Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. [Review]. J Antimicrob Chemother. 2005;55(Suppl 1):i5–21.
4. Brown IH. Advances in molecular diagnostics for avian influenza. Dev Biol (Basel). 2006;124:93–7.
5. Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA. 2006;295:891–4.
6. Klimov AI, Rocha E, Hayden FG, Shult PA, Roumillat LF, Cox NJ. Prolonged shedding of amantadine-resistant influenza A viruses by immunodeficient patients: detection by polymerase chain reaction-restriction analysis. J Infect Dis. 1995;172:1352–5.
7. Payungporn S, Chutinimitkul S, Chaisingh A, Damrongwantanapokin S, Buranathai C, Amonsin A, et al. Single step multiplex real-time RT-PCR for H5N1 influenza A virus detection. J Virol Methods. 2006;131:143–7.
8. Amonsin A, Payungporn P, Theamboonlers A, Thanawongnuwech R, Suradhat S, Pariyothorn N, et al. Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. Virology. 2006;344:480–91.
9. Chutinimitkul S, Bhattarakosol P, Srisuratanon S, Eiamudomkan A, Kongsomboon K, Damrongwatanapokin S, et al. H5N1 influenza A virus and infected human plasma. Emerg Infect Dis. 2006;12:1041–3.

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Laboratory Exposure to Influenza A H2N2, Germany, 2004–2005

To the Editor: From November 2004 to February 2005, a company contracted by the College of American Pathologists (CAP) sent influenza quality assurance samples containing live influenza A H2N2 viruses (A/Japan/305/57) to 3,748 international laboratories (*1*,*2*). Of these, 3,686 (98%) were located in Canada or the United States. In Germany, 6 laboratories received at least 1 of 3 samples, 2 for virus antigen detection and 1 for virus culture; all contained live virus and were formatted identically. No information on infectivity or virulence of the samples was available. Because of the absence of human-to-human influenza A H2N2 virus transmission since 1968, this situation provided the rare opportunity to investigate whether infections with this strain had occurred in any of the laboratories.

We used a standardized questionnaire to obtain from the laboratories information on when the CAP samples had been received, which of the 3 quality assurance specimens they contained, and how many employees had been involved in their handling. A second questionnaire was distributed to personnel in microbiology and virology departments. This elicited information regarding routine laboratory activities, contact with CAP samples, tasks performed in conjunction with handling of the samples, and any influenzalike symptoms (sudden onset of fever, cough, headache, and muscle pain) within the respective time frame. Persons who had worked in a receiving laboratory from September 1, 2004, to April 15, 2005, and had performed routine procedures in virology (defined as transport of samples, preparation of samples for diagnostic testing, antigen testing, nucleic acid amplification testing, and virus isolation) were eligible for the study. From May 4 to May 19, 2005, we visited the laboratories to interview supervisory personnel regarding routine work-up of samples and to collect blood from study participants for serologic testing.

The national reference laboratory for influenza at Robert Koch Institute performed serologic testing for antibodies against A/Singapore/1/57 (H2N2) virus by hemagglutination inhibition. We compared antibody titers of laboratory workers who worked with a CAP sample with those who did not. However, this comparison might not show a difference if (silent) virus transmission had occurred among laboratory staff. To exclude this possibility, we also compared titers of workers born before 1969 with those in a group of volunteers from Robert Koch Institute also born before 1969. Titers <10 were assigned a value of 1.

Of 47 laboratory workers, 18 either declined to participate or were excluded because they did not perform any of the defined routine procedures during the defined period. Thus, 29 (62%) workers were included in the study, of whom 14 (48%) reported having worked with CAP samples. Of these 14 workers, 12 (2 exclusively) transported samples and 11 (2 exclusively) prepared the samples; 9 (1 exclusively) performed antigen testing, 2 (0) performed nucleic acid amplification tests and 4 (0) performed virus isolation. Fourteen workers (48%) reported no contact with the samples, and 1 (3%) was unsure. None of the 29 participants reported any event that could have led to release of infectious material during the respective time frame, such as broken test tubes or dropped culture plates. Participating laboratories reported that all procedures were performed under appropriate hygienic and safety precautions. No person had ≥3 symptoms typical for influenzalike illness in the 4 days after having worked with a CAP sample.

Specific influenza A H2N2 antibody titers were determined in 25 study participants. None had a titer >80, two (8%) had a titer of 80, three (12%) had titers of 40, two (8%) had titers of 20, and the remaining 18 (72%) had titers ≤10. Three (21%) of 14 workers exposed to CAP samples and 4 (40%) of 10 who denied exposure had titers ≥20. All 7 were born before 1969. The geometric mean of titers of all participating workers born before 1969 did not differ significantly from that of the Robert Koch Institute employees ($p = 0.28$; Figure).

In summary, no evidence was found for laboratory infections with the influenza A H2N2 virus. The risk for laboratory-acquired influenza infections is unknown. Severe acute respiratory syndrome coronavirus and *Mycobacterium tuberculosis* are infectious agents whose transmission characteristics are similar to those of influenza. For severe acute respiratory syndrome coronavirus, laboratory-acquired infections are well documented (*3*,*4*). For *M. tuberculosis*, there are strong indications that they occur (*5*–*7*). From a public health perspective and in view of the current importance given to influenza and a possible pandemic, accurate characterization of the risk for influenza infections in laboratory settings is needed. The small number of persons included in this study limits the conclusions that can be drawn. Potentially exposed workers were

Figure. Titers of antibodies to influenza A H2N2 virus in laboratory personnel (n = 25; 13 born before 1969) and a comparison group born before 1969 (n = 32). The age listed is that in 2005. Titers <10 were assigned a value of 1.

presumably tested in other laboratories involved, but we are not aware of any publications to this end. The lack of evidence for laboratory-acquired infection with A H2N2 in our study suggests that the risk was low under controlled laboratory conditions. However, only a large-scale serologic study (which might still feasibly be undertaken) could further substantiate this finding.

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References

1. Eurosurveillance editorial team, Meijer A, Brown C, Paget J. Worldwide laboratory distribution of influenza A/H2N2 virus similar to 1957–58 pandemic strain, labs asked to destroy all samples immediately. Eurosurveillance. 2005;10. [cited 2006 Sep 15]. Available from http://www.eurosurveillance.org/ew/2005/050414.asp
2. World Health Organization. International response to the distribution of a H2N2 influenza virus for laboratory testing: risk considered low for laboratory workers and the public. 2005 [cited 2006 May 22]. Available from www.who.int/csr/disease/influenza/h2n2_2005_04_12/en/.
3. Normile D. Mounting lab accidents raise SARS fears. Science. 2004;304:659–61.
4. Lim PL, Kurup A, Gopalakrishna G, Chan KP, Wong CW, Ng LC, et al. Laboratory-acquired severe respiratory syndrome. N Engl J Med. 2004;350:1740–5.
5. Seidler A, Nienhaus A. Epidemiologie der berufsbedingten tuberkulose: ein literaturreview zu konventionellen studien. In: Nienhaus A, Brandenburg S, Teschler H, editors. Tuberkulose als berufskrankheit: ein leitfaden zur begutachtung. Landsberg (Germany): Ecomed Verlag; 2003. p. 142–69.
6. Jacobson JT, Orlob RB, Clayton JL. Infections acquired in clinical laboratories in Utah. J Clin Microbiol. 1985;21:486–9.
7. Kao AS, Ashford DA, McNeil MM, Warren NG, Good RC. Descriptive profile of tuberculin skin testing programs and laboratory-acquired tuberculosis infections in public health laboratories. J Clin Microbiol. 1997;35:1847–51.

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Methicillin-resistant *Staphylococcus aureus* in Cat and Owner

To the Editor: A 3-year-old, neutered male, domestic shorthaired cat was referred for treatment to a veterinary specialty clinic in San Francisco, California, with a 1-year history of multifocal patches of crusted and well-demarcated ulcers on the trunk. Initially, small crusts suspected to be associated with flea allergy and pyoderma were present; however, response was poor to multiple treatments, including repeated corticosteroid therapy and antimicrobial therapy with amoxicillin–clavulanic acid and enrofloxacin. The owner reported having skin abscesses and pneumonia 3 months earlier, although no microbiologic testing was performed.

Cytologic evaluation of exudate from the cat's lesions identified neutrophils and eosinophils with engulfed cocci. Leukocytosis with eosinophilia was found on a complete blood cell count. No notable abnormalities were present on thoracic radiograph, abdominal ultrasonograph, urinalysis, and tests for feline leukemia and immunodeficiency virus. Skin biopsy specimens were collected for histologic examination, and swabs of the exudates were submitted for bacterial culture. Histopathologic findings demonstrated ulcers and dermal granulation tissue with linearly arranged eosinophils, mast cells, neutrophils, and plasma cells between dense, homogeneous collagen bundles (sclerosing dermatitis). This pattern of inflammation is distinct from most staphylococcal infections of the skin, and it has been suggested that this uncommon histologic finding in cats is associated with methicillin-resistant staphylococcal infection (*1*).

Methicillin-resistant *Staphylococcus aureus* (MRSA) was isolated

from the skin lesions. Identification was confirmed by detection of penicillin-binding protein 2a (PBP2a) by latex agglutination test (PBP2′ Test Kit, Oxoid, Hants, UK). Typing was performed by *Sma*I pulsed-field gel electrophoresis as previously described (*2*), and the isolate was classified as the USA300 clone. Genes encoding production of the Panton-Valentine leukocidin (PVL) were identified by real-time PCR (*3*). The isolate was susceptible to chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin, but resistant to β-lactams, enrofloxacin, and erythromycin. After identification of MRSA in the cat, swabs of the anterior nares were collected from the owner and the cat, and MRSA was identified in specimens from both. All isolates were indistinguishable.

This is the first report of isolation of USA300 MRSA from a household pet. USA300 is a community-associated clone that has disseminated widely throughout North America and Europe (*4,5*) and is reaching epidemic proportions in many regions. MRSA is becoming an important cause of skin and soft tissue infection in persons in the community (*4,5*) and has also been implicated in invasive infections such as necrotizing pneumonia (*6*). This clone possesses genes for PVL production, which may be an important factor in its apparent virulence (*4,5*). Additional characterization of the isolates from this study was not performed; however, USA300 has previously been reported to carry staphylococcal cassette chromosome *mec* (SCCmec) type IVa and classified as sequence type 8 (ST8) by multilocus sequence typing (*4,5*).

Reports of MRSA infection and colonization in pets have increased dramatically in the past few years (*3,7–9*). Although this rise may be partially the result of increased testing and reporting, MRSA is definitely emerging in pet populations throughout the world. The role of pets in transmission of MRSA is still unclear; however, recent evidence suggests that MRSA can be transmitted between persons and their pets, in both directions (*9,10*). Reports of MRSA infection and colonization in pets have indicated that pets tend to be infected with isolates that are consistent with clones that are predominant in the human population in their area (*7–9*). Accordingly, USA100 accounted for initial isolations of MRSA in pets in North America (*9*). The similarity between pet and human isolates has led to speculation that pet MRSA is closely linked to human MRSA and that the source of MRSA in pets may often be colonized humans. If this is the case, it is not surprising that USA300 would emerge as a cause of disease in pets as it increases in prevalence in the human population. Considering the rapid dissemination of USA300 in humans in the United States, particularly in California, where it is the predominant community-associated clone, finding USA300 in a household pet in that state is not unexpected.

Because indistinguishable isolates were collected from the owner and the infected cat, MRSA likely was transmitted between species in the household. However, while it is tempting to assume that the owner was the source of infection because of the owner's previous history of a soft tissue infection, this cannot be definitively determined on the basis of the timing of sampling in this case.

MRSA appears to be emerging as an important veterinary and zoonotic pathogen, and the epidemiology of MRSA in household pets may take a parallel course to that in humans. Ongoing MRSA surveillance in animals is required, including proper testing of specimens from clinically affected animals and surveillance for colonization. The potential for transmission of this clone between humans and pets should also be evaluated to clarify its epidemiology and to facilitate development of measures to reduce household transmission.

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References

1. Ozaki K, Yamagami T, Nomura K, Haritani M, Tsutsumi Y, Narama I. Abscess-forming inflammatory granulation tissue with gram-positive cocci and prominent eosinophil infiltration in cats: possible infection of methicillin-resistant *Staphylococcus*. Vet Pathol. 2003;40:283–7.
2. Mulvey MR, Chui L, Ismail J, Louie L, Murphy C, Chang N, et al. Development of a Canadian standardized protocol for subtyping methicillin-resistant *Staphylococcus aureus* isolates using pulsed-field gel electrophoresis. J Clin Microbiol. 2001;39: 3481–5.
3. Rankin S, Roberts S, O'Shea K, Maloney D, Lorenzo M, Benson CE. Panton Valentine leukocidin (PVL) toxin positive MRSA strains isolated from companion animals. Vet Microbiol. 2005;108:145–8.
4. Roberts JC, Krueger RL, Peak KK, Veguilla W, Cannons AC, Amuso PT, et al. Community-associated methicillin-resistant *Staphylococcus aureus* epidemic clone USA300 in isolates from Florida and Washington. J Clin Microbiol. 2006;44: 225–6.
5. Tenover FC, McDougal LK, Goering RV, Killgore G, Projan SJ, Patel JB, et al. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J Clin Microbiol. 2006; 44:108–18.
6. Frazee BW, Salz TO, Lambert L, Perdreau-Remington F. Fatal community-associated methicillin-resistant *Staphylococcus aureus* pneumonia in an immunocompetent young adult. Ann Emerg Med. 2005;46:401–4.
7. Loeffler A, Boag AK, Sung J, Lindsay JA, Guardabassi L, Dalsgaard A, et al. Prevalence of methicillin-resistant *Staphylococcus aureus* among staff and pets in a small animal referral hospital in the UK. J Antimicrob Chemother. 2005;56:692–7.
8. Rich M, Roberts L. Methicillin-resistant *Staphylococcus aureus* isolates from companion animals. Vet Rec. 2004;154:310.

9. Weese JS, Dick H, Willey BM, McGeer A, Kreiswirth BN, Innis B, et al. Suspected transmission of methicillin-resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. Vet Microbiol. 2006;115: 148–55.
10. van Duijkeren E, Wolfhagen MJHM, Box ATA, Heck MEOC, Wannet JB, Fluit AC. Human-to-dog transmission of methicillin-resistant *Staphylococcus aureus*. Emerg Infect Dis. 2004;10:2235–6.

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Community-associated Methicillin-resistant *Staphylococcus aureus*, Colombia

To the Editor: Methicillin-resistant *Staphylococcus aureus* (MRSA) is an established nosocomial pathogen worldwide but more recently has emerged as a highly virulent organism in the community, particularly in the United States (*1*–*3*). In Latin America, community-associated MRSA (CA-MRSA) has only been described in the southern area of the continent (Uruguay and Brazil) (*4*,*5*). No reports from the Andean region are available. We describe 2 cases of CA-MRSA causing soft-tissue infections (1 severe) in Colombia.

The first case was in a 19-year-old man with a history of trauma to the left side of his body 1 week before admission after a fall. On admission, he complained of 2 days of fever, malaise, erythema and induration in the left hemithorax extending to the left thigh, and purulent secretion from an excoriation on the anterior aspect of the left thigh. He had no previous medical history. No previous hospitalizations or antimicrobial drug prescriptions were documented, nor did he report relatives with history of recent hospitalizations. Vital signs at admission were normal except for fever (39°C), and physical examination showed induration and erythema in the region of left hemithorax extending to the thigh, with an area of excoriation in the same thigh with purulent discharge. Laboratory evaluation showed a leukocyte count of 23.1×10^9/L (86% neutrophils with 2% band forms) and elevated C-reactive protein levels.

The patient was hospitalized. Because necrotizing fascitis was suspected, intravenous ampicillin-sulbactam (12 g per day) was started, and surgical evaluation was requested. The patient underwent surgical debridement of the left thigh, left hemiabdomen, and hemithorax, which confirmed the diagnosis of necrotizing fascitis. Intravenous vancomycin (1 g every 12 h) was added to the regimen, and the patient was transferred to the intensive care unit. After several surgical debridements, the patient underwent placement of cutaneous-muscle grafts. He was discharged from the hospital without complications after completing 14 days of antimicrobial agents.

The second case involved a 53-year-old man with no history of previous hospitalizations who reported to the emergency department with a 3-day history of fever, pain, swelling, and warm sensation on the posterior aspect of the left thigh. A diagnosis of cellulitis was made, and cephalexin (500 mg every 6 h) and gentamicin (160 mg intramuscularly every 24 h) were administered for 7 days without improvement. He returned to the hospital with worsening symptoms, an area of induration of 4×4 cm in the thigh, and purulent discharge. Drainage of the lesion was performed, and oral trimethoprim and sulfamethoxazole (160 and 800 mg, respectively, every 12 h) was started. His clinical signs and symptoms completely resolved after 7 days of therapy.

Tissue culture from secretions from both patients showed gram-positive cocci in clusters on the Gram stain, and subsequent cultures yielded MRSA. Species identification and presence of the *mecA* gene were confirmed by PCR, as described previously (*6*). MICs were determined by using the agar diffusion test, according to Clinical and Laboratory Standards Institute recommendations (*7*). Both organisms were susceptible to vancomycin, teicoplanin, chloramphenicol, linezolid, ciprofloxacin, gentamicin, and rifampin. The isolate from the second patient was resistant to erythromycin and susceptible to clindamycin, exhibited the M phenotype on the double-disk diffusion assay (D test), and harbored the *msrA* gene, encoding an efflux pump (*8*). In contrast, the first isolate was susceptible to both erythromycin and clindamycin and resistant to tetracycline (MIC >64 μg/mL). Because infections caused by CA-MRSA isolated elsewhere are associated with the presence of the *lukF* gene encoding the Panton-Valentine leukocidin toxin and the staphylococcal chromosome cassette *mec* (SCC*mec*) type IV, the presence of both was evaluated by PCR, as described previously (*9*). Both isolates were positive for *lukF* and harbored the SCC*mec* type IV.

The molecular epidemiology of healthcare-related MRSA in Colombia has changed during the past 3 years (*10*), but no reports of CA-MRSA had emerged. We believe these to be the first reports of CA-MRSA in Colombia with similar characteristics to those reported elsewhere. No risk factors associated with healthcare-associated MRSA were found in either of these patients, and the patients were not epidemiologically

related. The first case involved a severe soft-tissue infection associated with CA-MRSA. Clinicians should be aware of the circulation of CA-MRSA in Colombia.

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References

1. Vandenesch F, Naimi T, Enrigth MC, Lina G, Nimmo GR, Herffernan H, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentin leuckocidin genes: worldwide emergence. Emerg Infect Dis. 2003;9:978–84.
2. Maltezou HC, Giamarellou H. Community-acquired methicillin-resistant *Staphylococcus aureus* infections. Int J Antimicrob Agents. 2006;27:87–96.
3. Zetola N, Francis JS, Nuermberger EL, Bishai WR. Community-acquired methicillin-resistant *Staphylococcus aureus:* an emerging threat. Lancet Infect Dis. 2005;5: 275–86.
4. Ma XX, Galiana A, Pedreira W, Mowszovicz M, Christophersen I, Machiavello S, et al. Community-acquired methicillin-resistant *Staphylococcus aureus*, Uruguay. Emerg Infect Dis. 2005;11:973–6.
5. Ribeiro A, Días C, Silva-Carvalho MC, Berquo L, Ferreira FA, Santos RN, et al. First report of infection with community-acquired methicillin-resistant *Staphylococcus aureus* in South America. J Clin Microbiol. 2005;43:1985–8.
6. Martineau F, Francois J, Picarda LG, Paul H, Roya MO, Michel G; ESPRIT Trial. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. J Antimicrob Chemother. 2000;46:527–34.
7. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Document M100-S15. Wayne (PA): The Institute; 2005.
8. Leclercq R. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin Infect Dis. 2002;34:482–92.
9. Oliveira DC, de Lencastre H. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 2002;46:2155–61.
10. Cruz C, Moreno J, Renzoni A, Hidalgo M, Reyes J, Schienzel J. el al. Tracking methicillin-resistant *Staphylococcus aureus* clones in Colombian hospitals over 7 years (1996–2003): emergence of a new dominant clone. Int J Antimicrob Agents. 2005;26:457–62.

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Live Nativity and Brucellosis, Sicily

To the Editor: Worldwide, brucellosis remains a major zoonosis and an important cause of travel-associated illness (*1*). Brucellosis is transmitted to humans through the consumption of infected, unpasteurized, animal-milk products; direct contact with infected animal parts; or inhalation of infected aerosolized particles. We report an outbreak of brucellosis in a small village of the Ionic coast of Messina province (eastern Sicily).

In 2003, health authorities in the Messina province were notified of 29 cases of brucellosis; 18 of the patients were members of 9 different families. All patients had observed a Nativity pantomime that used live animals and was organized by the local population. Nativities in Sicily last ≈1 month, during which the sheep are milked, cheese and ricotta are produced, and these products are sold or offered fresh to tourists. All 29 patients had consumed dairy products: tuma cheese by 29 (100%) and tuma and ricotta by 16 (55%). No other risk factors for brucellosis were reported. Symptoms appeared after a median of 45 days (range 30–70). Eight patients were children (3 male), and 21 were adults (10 male). The median age of the children was 10.5 years (range 6–13) and of the adults, 42 years (range 16–67). Hospitalization was required for 5 patients. For 2 adults, brucellosis was complicated by spondylitis.

The real extent of the outbreak was likely large because in Sicily ≈60% of cases may go unreported. Furthermore, we report only the cases that occurred in the villages of Messina province and that were reported to health authorities; but tourists from many other areas in Italy and some from outside Italy generally attend such events. Southern Italy has commonly been implicated as a venue for travel-associated brucellosis (*2*).

In Italy, the overall incidence of brucellosis has gradually declined in the past 30 years, especially in northern Italy, where the disease is now reported only sporadically. This trend, however, has led to an increase in the percentage of total cases in Italians reported from the southern provinces of Calabria, Campagna, Puglia, and Sicily; of the 520 cases reported in 2003, 488 (93.8%) were reported from 4 southern regions, compared with 63.7% in 1994. Sicily alone reported 57.6% of the 2003 cases and for the past decade has had an average annual incidence of >100 cases per million (*1*,*3*). The disease is almost always caused by *Brucella melitensis* (*4*). The southern localization of the disease in Italy is obviously related to the relative high prevalence of infections in sheep and goats (*5*). Ovine and caprine population density is higher in the southern regions of Italy than in the rest of the country (*6*).

The Italian brucellosis eradication plan consists of a test-and-slaughter practice. However, in Sicily a vaccination campaign with *B. melitensis* Rev.1 strain has been started. In 2003, ≈99% of the stock farms were tested, and ≈18% of them were infected (*3*).

The major problems reside with small flocks that undergo frequent transhumance (seasonal movement of herds between regions with different climates) in isolated regions where testing by veterinarians is difficult or avoided by the owners (a typical drawback of test-and-slaughter practices).

Technically, ricotta is not a cheese, but rather is a cheese by-product. The name "ricotta" means cooked again, referencing the production method. Ricotta is made from whey drained from tuma, provolone, and other cheeses. Heat is then used to separate, by precipitation, the remaining albumin from the whey left after making lactic acid/rennet-precipitated cheeses. It is eaten as is or used for food seasoning (e.g., classic Italian lasagna and ravioli). A cream made of sieved ricotta and sugar is used to prepare many desserts, like cannoli and cassata cake. Being cooked 2 times, ricotta should not contain viable *Brucella* organisms; however, shepherds sprinkle fresh milk on wicker baskets to refresh the ricotta they contain, thereby contaminating the product.

Tuma is a typical Sicilian fresh cheese made from sheep's milk. It has a cylindrical appearance and is sold fresh, no more than 2 days old. It has no crust, and the dough is white or ivory-white without holes. The texture is very soft, tender, and wet. It is generally served with ham, wines, and fruits as a table cheese.

Tuma cheese should be considered as the major vehicle of *B. melitensis* infection in Sicily. Although most similar dairy products produced in Sicily are derived from organized dairy companies and have been pasteurized, traditional delicacies from small villages may still cause brucellosis outbreaks.

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References

1. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet Infect Dis. 2006;6:91–9.
2. Chanet V, Gourdon F, Baud O, Beytout J, Romazko JP. *Brucella melitensis* in a married couple after a trip through Sicily. South Med J. 2005;98:843–4.
3. Ministry of Health, Italy. Results of epidemiological research. [cited 2006 Jul 18]. Available from http://www.ministerosalute.it/promozione/malattie/datidefcons.jsp
4. Caporale V, Nannini D, Giovannini A, Morelli D, Ramasco M. Prophylaxis and control of brucellosis due to *Brucella melitensis* in Italy: acquired and expected results. In: Prevention of brucellosis in the Mediterranean countries. Proceedings of the International Seminar of the International Center for Advanced Mediterranean Agronomic Studies (CIHEAM), the Commission of the European Communities, and the Ministry of Agriculture and Fisheries. Valletta (Malta): CIHEAM, 1992. p. 127–45.
5. De Massis F, Di Girolamo A, Petrini A, Pizzigallo E, Giovannini A. Correlation between animal and human brucellosis in Italy during the period 1997-2002. Clin Microbiol Infect. 2005;11:632–6.
6. Caracappa S. Livestock production and animal health in Sicily, Italy. Parassitologia. 1999;41(Suppl 1):17–23.

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Foodborne Pathogens: Microbiology and Molecular Biology

Pina M. Fratamico, Arun K. Bhunia, and James L. Smith, editors

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Foodborne pathogens can create a considerable amount of work at state and local health departments. Between foodborne outbreaks, restaurant inspections, environmental testing, botulism reports, customer complaints, and confirmation of isolates referred for testing, many health department resources are directed toward these pathogens and preventing illness from them. Moreover, the mass media are increasingly interested in food safety, particularly after large, multistate outbreaks caused by *Escherichia coli* O157:H7 and *Salmonella*, among other pathogens, and increasing public interest in raw and unpasteurized foods that are perceived as more natural or healthy. The audience for Foodborne Pathogens: Microbiology and Molecular Biology appears to be public health practitioners working on epidemiologic, environmental, and laboratory aspects of foodborne illness.

One of the book's strengths is that it attempts to include reference material on epidemiology and on the molecular and microbiologic aspects of the various pathogens. However, as the title suggests, the emphasis is on molecular and microbiologic aspects, and much of the information is extremely technical and primarily for the laboratory scientist. The book includes a range of food pathogens, from bacteria and viruses to mycotoxins. The primary omission is bovine spongiform encephalopathy. Chronic wasting disease is included briefly in a chapter on potential food pathogens, which makes the omission of bovine spongiform encephalopathy all the more striking.

In addition to separate chapters on individual pathogens or groups of pathogens, the book covers laboratory issues, including animal and cell culture models, molecular approaches for detection, and stress responses of foodborne pathogens. Other chapters are based on more sensational topics, such as bioterrorism and food, although this chapter discusses the subject in general terms. In a chapter on biosensor-based detection of foodborne pathogens, the authors conclude, not convincingly, that biosensors will soon be as widespread as glucose kits and home pregnancy tests.

Overall, the book is a good reference for health departments, especially the chapters on individual pathogens. However, the book could have used stronger editorial oversight. Books like this one, in which experts in highly specialized fields are each invited to write a chapter, will by their very nature lack an overriding point-of-view, but at the very least, the book should have had a strong introduction to put the content in context.

A large number of pathogens have emerged or been identified in the past 30 years, and a great deal of media attention is given to food-related illness. This book appears to be aimed at industrialized countries, despite the perception that the food supply in these countries is safe. Because much food is imported and exported throughout the world, including to and from industrialized nations, some basic discussion of the extent of foodborne illness in different parts of the world, and the resulting risk to the overall food supply, would have helped to frame the need for the book and the resources many health departments are putting toward foodborne illness.

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Red Book: 2006 Report of the Committee on Infectious Diseases, 27th Edition

L.K. Pickering, editor; C.J. Baker, S.S. Long, and J.A. McMillan, associate editors

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The 27th edition of the 2006 Report of the American Academy of Pediatrics (AAP) Committee on Infectious Diseases, known to most clinicians as "The Red Book," is considered the "Bible" of pediatric infectious diseases. In addition to providing an updated and exhaustive summary of the clinical manifestations, etiology, epidemiology, diagnostic tests, treatment, isolation, and control measures for >200 pediatric infectious diseases, this reference discusses a number of related topics, including management. With >350 liaisons and collaborators from the Centers for Disease Control

and Prevention, the Food and Drug Administration, the National Institutes of Health, the Canadian Paediatric Society, the World Health Organization, and others, the 12-member 2004–2006 Committee on Infectious Diseases issued the current edition, which reflects the state of the art at the time of publication and is updated every 3 years.

The Red Book began as an 8-page mimeographed report assembled in 1937 by the Committee on Immunization and Therapeutic Procedures for Acute Infectious Diseases (currently the Committee on Infectious Diseases) and titled "Immunization Procedures." A revision issued in 1938 in pamphlet form was actively sought, and annual issues became more comprehensive, with the addition of 76 pages reflecting the increase in therapeutic antimicrobial drug options in the ensuing 10 years.

The current 992-page 27th edition has newly added sections, including key developments in combination vaccines, the 2006 AAP standards for child and adolescent immunization practices, parental refusal to vaccinate, updates on prevention of mosquitoborne infections, and updated information on emerging infectious diseases and pathogens, including *Baylisascaris*, metapneumovirus, West Nile virus, coronaviruses, pertussis, tuberculosis, and pneumococcal, meningococcal, and varicella infections. Expanded discussions of drug interactions as well as the revised American Heart Association recommendations for the prevention of bacterial endocarditis are presented. Many website resources have been added throughout the text to provide the reader with links to expanded information about the topic.

The text is organized into 5 sections with a comprehensive list of appendixes. Section 1 reviews active and passive immunization and provides a practical discussion of numerous aspects of vaccine administration, including vaccine shortages, reporting of vaccine-preventable diseases, and parental misconceptions about vaccinations. Section 2 provides recommendations for care of children in special circumstances, including the topic areas of biological terrorism, children in out-of-home child care, infection control, and medical evaluation of internationally adopted children. This section serves as a comprehensive resource for both general and infectious diseases pediatricians. The 3rd section, an alphabetical summary of infectious diseases, comprises the bulk of the text. Sections 4 and 5 address the expanding category of antimicrobial agents, therapy, and prophylaxis, including guidelines and indications for their appropriate use.

The area of infectious diseases is rapidly emerging and changing, so the guidelines presented in the current edition may have undergone updating and revision following publication. Therefore, readers are urged to monitor updated recommendations issued by the Committee on Infectious Diseases on the Red Book Online website (http://www.aapredbook.org). This site also lists errata from the current edition and allows readers to register to be notified when new errata are posted, when new policy statements are issued, and when site updates and new features are added. Readers may also register for a customized citation/keyword alert.

The 27th edition of the Red Book is a vital resource for adult and pediatric infectious disease practitioners as well as general practitioners and is considered by many the quintessential resource and reference for clinical practice.

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Roelandt Savery (1576–1639) (detail). The Garden of Eden (1618). Oil on wood (55 cm × 107 cm). National Gallery, Prague, Czech Republic

Spatial Distribution and the Animal Landscape

Polyxeni Potter*

Peace and harmony reign in Roelandt Savery's The Garden of Eden, on this month's cover of Emerging Infectious Diseases. Animals sprawled across the expansive landscape move languidly, unaware of any danger to them from each other or their surroundings, their graceful contours and anatomic details of greater interest to the artist than their natural temperament. The pastoral scene lends itself to the paradoxical rendition, common in Flanders during Savery's time, of the imaginary put in realistic terms.

Flanders (in today's Belgium) was the region of Europe known for great cultural achievements, particularly in the Middle Ages and during the 16th and 17th centuries, when Flemish, also known as Netherlandish, art flourished. Landscape painting, which had been merely the setting for religious scenes, became important in its own right in the art centers of Antwerp and Brussels, and when Joachim Patinir, one of the first to create stand-alone landscapes, was praised by Albrecht Dürer as a "good landscape painter," the term landscape was elevated for the first time outside the context of Italian art (*1*). The genre was brought to new heights by such masters as Jan Bruegel the Elder and Peter Paul Rubens.

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Contemporary of these great masters and the best-known in a family of artists, Savery was born in the Dutch village of Kortrijk and grew up in Amsterdam, where he studied under his brother Jacob and artist Hans Bol. He traveled widely and worked for Henry IV in France and Emperor Matthias in Vienna, but his stay in Prague, where he was painter and etcher of landscapes, animals, and still lifes in the court of Rudolf II von Habsburg, produced the work for which he is most remembered (*2*).

Rudolf, emperor of the Holy Roman Empire and king of Bohemia and Hungary, was known for his eccentricity, one sign of which was his famed menagerie of exotic animals. Obsessive collector of unusual objects and lover of art and architecture, as well as science, the offbeat emperor surrounded himself with such artists as Bartholomeus Spranger and Giuseppe Arcimboldo, and with scientists, among them astronomers Tycho Brahe and Johannes Kepler (*2*). This period saw a rise in the study of mathematics, optics, physics, biology, and the development and propagation of hybrid flowers as scientific and economic pursuits.

Savery's peers in a thriving art community increasingly specialized, producing landscapes unlike any up to this time. He dabbled with and influenced several genres. His spectacular views of precipitous rocks and waterfalls influenced Dutch landscape painting. His much sought-after

floral still lifes, along with those of Jan Brueghel the Elder, Ambrosius Bosschaert the Elder, and Gillis van Coninxloo, which they surpassed in tonal quality and realism, mark the beginning of the great age of Dutch flower painting (*3*).

The first Dutch artist to do so, Savery turned his talented hand to animal painting. Riding a strong interest in zoology at the Habsburg court and with full access to Rudolf's menagerie, he became a leader in this genre, painting and drawing a great variety of animals: pelicans, ostriches, camels, and the now extinct dodo, which he immortalized in several works. One of these, in which the dodo was the main subject, was presented to the British Museum in 1759 as painted "in Holland from the living bird" (*4*). Once he was paid 700 guilders, then an enormous sum, to paint "all the animals of the air and earth" (*5*). Soon his mythologic scenes became thinly disguised opportunities to paint more species. His favorite subjects were Orpheus charming the beasts, Noah's Ark, and scenes of Eden.

The Garden of Eden is an animal painting of the style initiated by Savery amidst increased interest in biologic research and rare creatures. The biblical narrative is only the vehicle for displaying the water buffalo, the dromedary, and all manner of unusual beasts. "Swarms of living creatures" of the mountain or the prairie, in any color, texture, shape, and form, inhabit the unlikely locale, predator frolicking with prey, desert beast with tropical. In a virtuoso display of spatial depth, Savery places Adam in the distant horizon, under the Tree of Knowledge, naming the animals "Whatsoever Adam called every living creature, that was the name thereof" (Gen. 2:19).

Naming the animals has long fascinated humans, from Aristotle to Linnaeus. And Adam's awesome task has not been completed, as indeed no one has been able to name or paint "all the animals of the air and earth." New species continue to be found (e.g., recently *Mus cypriacus* [*6*]). Others are dead as the dodo.

Like other interpretations of Eden, Savery's is not so much a geographic location as an idealized landscape. And while his beasts' exotic perfection was painted with meticulous attention to detail, the realism extended only to form, species variation clearly at odds with normal species distribution. Today, the imaginary in Savery's painting has become real. Dissimilar and diverse species from around the globe *are* mingled, and not just for a photo opportunity.

In collections of exotic animals, as well as in homes, parks, and the wild, far from their places of origin, animals wander the globe imported as pets, contraband cargo on board ship or plane, or in natural habitat claimed by urban and agricultural development. And contrary to notions of Eden, coexistence has been all but peaceful and harmonious. From the merger of bats, pigs, and people (Nipah virus) to the mingling of African rodents with their North American relatives (monkeypox), from AIDS to SARS to avian flu, the changing landscape of animal habitat is changing the geography and ecology of disease transmission.

References

1. Whinney M. Early Flemish painting. London: Faber & Faber, Ltd; 1968.
2. Evans RJW. Rudolf II and his world: a study in the intellectual history, 1576–1612. Oxford: Clarendon Press; 1973.
3. Bernt W. The Netherlandish painters of the seventeenth century. Oxford: Phaidon; 1948.
4. Roelandt Savery [cited 2006 Oct 26]. Available from http://www.nhm.ac.uk/nature-online/online-ex/art-themes/caught_in_oils/more/doto_more_info.htm
5. Roelandt Savery [cited 2006 Oct 26]. Available from http://www.figgeartmuseum.org/SiteDefault.aspx?PageID=30&nt=14&LastName=S
6. New mouse find is "living fossil." [cited 2006 Oct 30]. Available from http://newsvote.bbc.co.uk/mpapps/pagetools/print/news.bbc.couk/1/hi/sci/tech/6043648.s

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the January issue for the following topics:

Epidemics after Natural Disasters

Wildlife, Exotic Pets, and Emerging Zoonoses

Emergence of G2P[4] and G12P[6] Rotaviruses in Bangladesh

Elimination of Arctic Variant of Rabies in Red Foxes, Toronto, Ontario, Canada

Vaccine Effectiveness and Mumps Outbreak, England, 2004–2005

Donated Blood and Spread of Variant Creutzfeldt-Jakob Disease

Assessment of Human Papillomavirus Vaccination Strategies

Multidrug-resistant *Acinetobacter* Infection

Clusters of Hantavirus Infection, Southern Argentina

Emergence of Arctic Rabies Lineage in India

Spread of *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest, USA

Dispersal Mechanisms of *Cryptococcus gattii*, British Columbia, Canada

Primary *Pneumocystis* Infection in Infants Hospitalized with Acute Respiratory Tract Infection

Nor98 Cases in France and Norway

Complete list of articles in the January issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

December 11–12, 2006
1st Annual Alliance for the Prudent Use of Antibiotics World Congress
Hyatt Regency Hotel
Boston, MA, USA
http://www.apua.org

February 23–25, 2007
IMED 2007: International Meeting on Emerging Diseases and Surveillance
Vienna, Austria
Contact: info@isid.org or
617-277-0551 voice
617-278-9113 fax
http://imed.isid.org/

March 7–9, 2007
6th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2007)
Raffles City Convention Centre
Singapore
http://www.isaar.org

March 20–23, 2007
ISOPOL XVI: 16th International Symposium on Problems of Listeriosis
Marriott Riverfront Hotel
Savannah, GA, USA
Contact: 240-485-2776
http://www.aphl.org/conferences/ISOPOL.cfm

April 30–May 2, 2007
10th Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- ★ New infections resulting from changes or evolution of existing organisms.
- ★ Known infections spreading to new geographic areas or populations.
- ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
   - ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

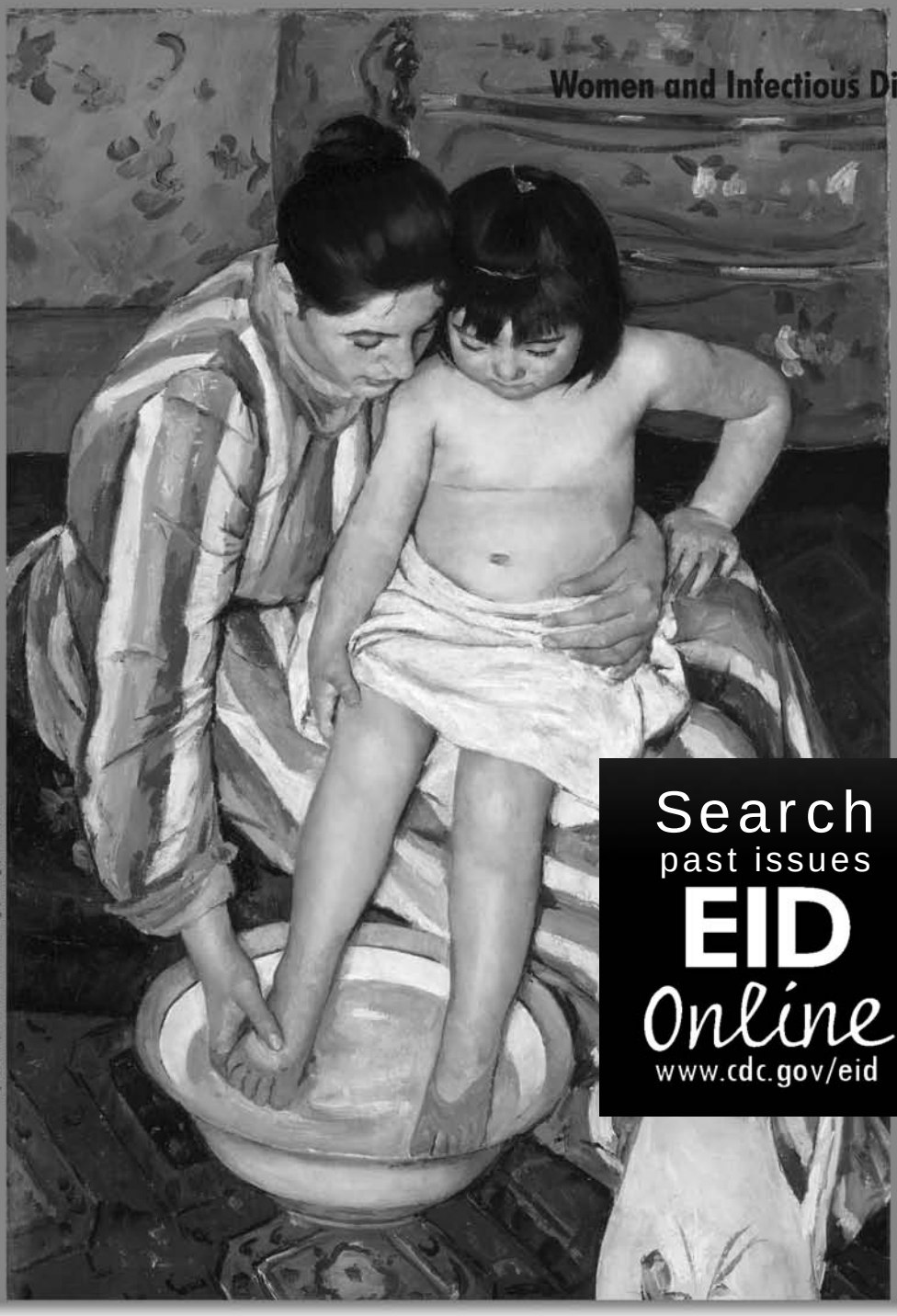
2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - ★ Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - ★ Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - ★ Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

EID Online www.cdc.gov/eid

November 2006



Women and Infectious Diseases

Reproduction, The Art Institute of Chicago, Chicago, Illinois, USA

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EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.