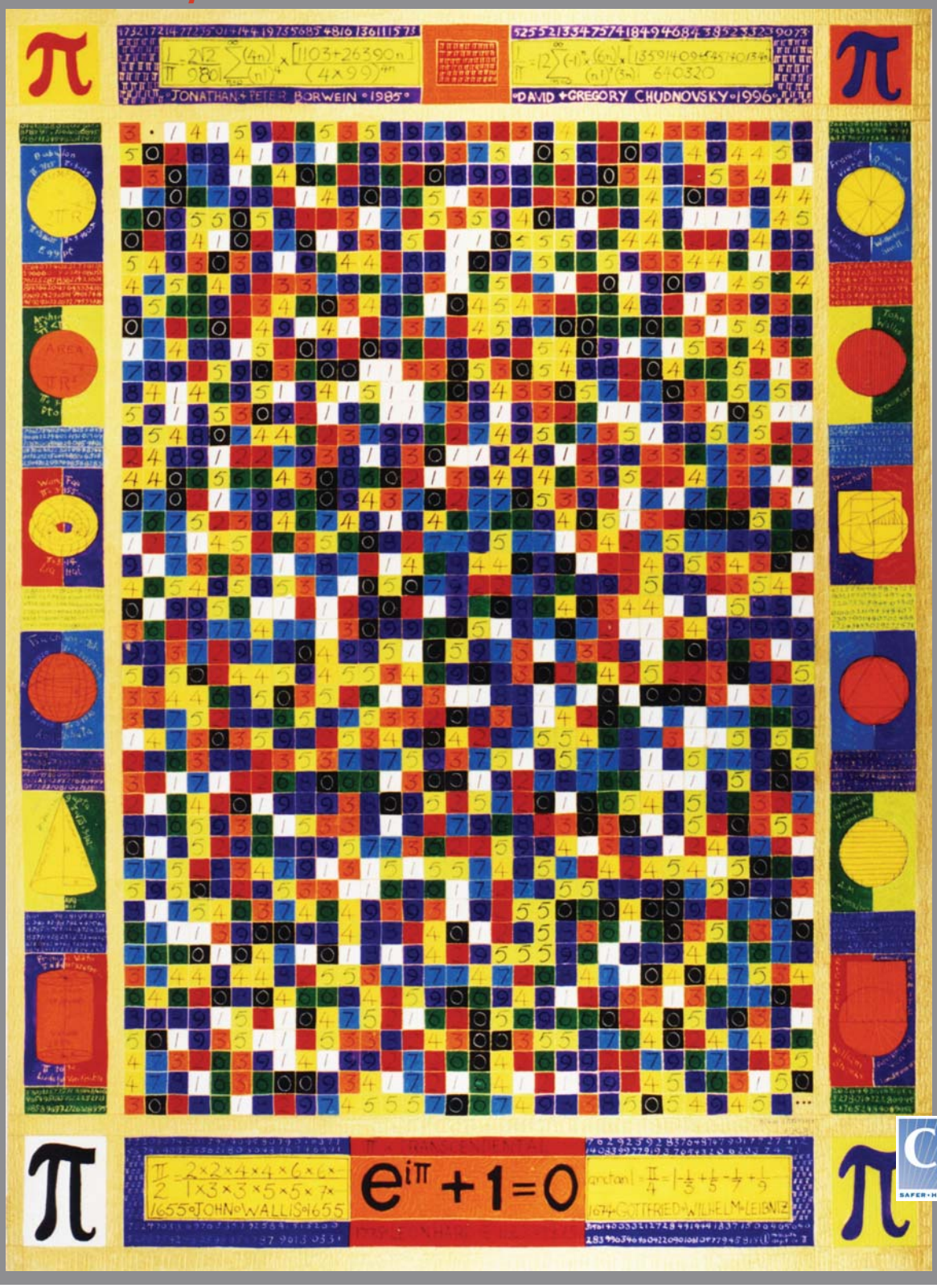
Vol 14, No 7, July 2008

EMERGING INFECTIOUS DISEASES®

EID Online www.cdc.gov/eid

July 2008

Central Nervous System



Used with permission of Robert A. Adams

EMERGING INFECTIOUS DISEASES

Pages 1013–1186

EMERGING INFECTIOUS DISEASES

www.cdc.gov/eid

Emerging Infectious Diseases
Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

EMERGING INFECTIOUS DISEASES

July 2008

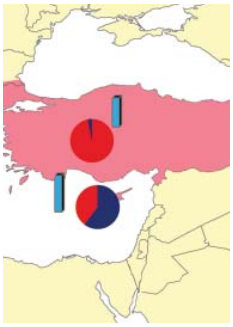
On the Cover

Anne Adams (1940–2007)
Pi (1998)
Gouache on paper.
Used with permission
of Robert A. Adams

About the Cover p. 1183

Perspective

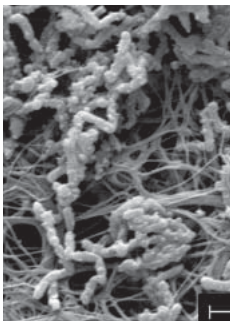
Synopsis



p. 1015

Research

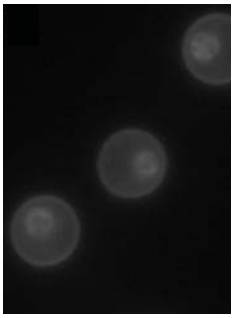
p. 1049



CME Activity

EMERGING INFECTIOUS DISEASES
July 2008

Dispatches



p. 1106

p. 1113

Another Dimension

Letters

Book Review

News & Notes

Online Reports

Spread of Vector-borne Diseases and Neglect of Leishmaniasis, Europe

Jean-Claude Dujardin,* Lenea Campino,† Carmen Cañavate,‡ Jean-Pierre Dedet,§ Luigi Gradoni,¶ Ketty Soteriadou,# Apostolos Mazeris, Yusuf Ozbel,†† and Marleen Boelaert***

The risk for reintroduction of some exotic vector-borne diseases in Europe has become a hot topic, while the reality of others is neglected at the public health policy level. Leishmaniasis is endemic in all southern countries of Europe, with ≈700 autochthonous human cases reported each year (3,950 if Turkey is included). Asymptomatic cases have been estimated at 30–100/1 symptomatic case, and leishmaniasis has up to 25% seroprevalence in domestic dogs. Even though leishmaniasis is essentially associated with *Leishmania infantum* and visceral leishmaniasis, new species, such as *L. donovani* and *L. tropica*, might colonize European sand fly vectors. Drug-resistant *L. infantum* strains might be exported outside Europe through dogs. Despite this possibility, no coordinated surveillance of the disease exists at the European level. In this review of leishmaniasis importance in Europe, we would like to bridge the gap between research and surveillance and control.

In August through September of 2007, a chikungunya outbreak occurred in the province of Ravenna, Italy (*1*). The risk for reintroduction of vector-borne diseases in Europe as a consequence of global warming was highlighted, although long-distance tourism, travel, and trade could also play major roles in the transcontinental transport of microorganisms (*2*). The European Centre for Disease Control is currently assessing the magnitude and importance of vector-borne diseases in Europe, focusing on Lyme disease, tick-borne encephalitis, leptospirosis, malaria, plague, tularemia, viral hemorrhagic fevers, hantavirus, and West Nile fever. Concern about the impact of global warming and the spread of arthropod-borne diseases and other infectious agents in Europe is justifiable. However, existing autochthonous vector-borne infections should not be forgotten or ignored, which may be the case, as illustrated here for leishmaniasis.

*Instituut voor Tropische Geneeskunde, Antwerp, Belgium; †Instituto de Higiene e Medicina Tropical, Lisbon, Portugal; ‡Instituto de Salud Carlos III, Madrid, Spain; §Université Montpellier 1, Montpellier, France; ¶Istituto Superiore di Sanità, Rome, Italy; #Hellenic Pasteur Institute, Athens, Greece; **National Reference Laboratory for Animal Health, Nicosia, Cyprus; and ††Ege University Medical School, Bornova, Izmir, Turkey

DOI: 10.3201/eid1407.071589

Leishmaniasis in Europe

Leishmaniasis is a major vector-borne disease, which is endemic to 88 countries and is the only tropical vector-borne disease that has been endemic to southern Europe for decades. In southern Europe, most of the reported cases are due to zoonotic visceral leishmaniasis (VL), which is the most dangerous form and is lethal when untreated. Cutaneous leishmaniasis (CL), which is more benign than VL, is also present. Incidence of leishmaniasis in humans is relatively low, ranging from 0.02/100,000 to 0.49/100,000 (8.53/100,000 including Turkey). We estimate that this corresponds to a total of ≈700 reported new cases per year for southern European countries (3,950 if Turkey is included; Table and Figure). However, autochthonous leishmaniasis appears not to be limited to the Mediterranean region anymore. It has spread northward, as shown by the recent reports of indigenous VL cases in northern Italy and southern Germany (*8*,*9*).

However, these numbers are misleading for several reasons. First, data from patients infected in southern Europe, but diagnosed elsewhere, are not taken into consideration. For instance, a leishmaniasis reference center established on a voluntary basis in Germany identified within 2 years 70 cases of leishmaniasis. Of the 27 VL case-patients, most

Table. Leishmaniasis situation in 7 disease-endemic countries of Europe (including Turkey)*

Country	Human leishmaniasis				Canine leishmaniasis
	Notification status	Current information from reference centers (2000–2006)	VL + CL incidence x 100,000†	Imported cases (VL + CL)	
Portugal‡	Compulsory for VL	≈22 VL cases/y recorded at IHMT	0.07–0.17	≈2 cases/y recorded at IHMT	Average 20% seroprevalence in disease-endemic areas (*3*)
Spain§	Compulsory in 12/17 autonomous communities; 20%–45% underreporting for VL, ≈100% for CL (*4*)	≈100 VL cases/y recorded by National Epidemiologic Surveillance Network, RENAVE	0.18–0.29	≈5 cases/y recorded at ISCIII	Average 8.5% seroprevalence (*5*)
France¶	Not compulsory, but spontaneous reports at UMON	≈24 VL + CL cases/y reported at UMON	0.02–0.19	≈65 cases/y recorded at UMON	Seroprevalence in disease-endemic areas of southern France 4%–20%#
Italy**	Compulsory for both VL and CL, but CL underreported	≈200 VL cases/y recorded at ISS; ≈300 CL cases/y estimated by ISS	0.15–0.38	≈8 cases/y recorded at ISS	Average 15% seroprevalence in peninsular Italy; average 2% seroprevalence in continental Italy (*6*)
Greece††	Compulsory for both VL and CL, but underreported	≈21 VL cases/y notified	0.06–0.49	Unknown	Average seroprevalence 25% in disease-endemic areas (*7*)
Cyprus‡‡	Compulsory for both VL and CL, but underreported	5 VL + CL cases recorded in 2006	0.25–0.47	Unknown	Average seroprevalence 20% in disease-endemic areas
Turkey§§	Compulsory for both VL and CL	≈37 VL cases/y and ≈2,300 CL cases/y notified	1.6–8.53	Unknown	Average 15.7% seroprevalence

*Authors' institutions are national reference laboratories for leishmaniasis diagnosis and surveillance and rely on consolidated countrywide networks of collaborating clinical health centers. Diagnosis records are cross-checked with case notifications to provide more realistic figures and estimates. VL, visceral leishmaniasis; CL, cutaneous Leishmaniasis; WHO, World Health Organization.
†WHO-EURO, WHO Europe, 1996–2005; http://data.euro.who.int/CISID.
‡Instituto de Higiene e Medicina Tropical (IHMT), Lisbon, Portugal.
§Instituto de Salud Carlos III (ISCIII), Madrid, Spain.
¶Université de Montpellier (UMON), data from Centre National de Référence des Leishmania, Montpellier, France.
#Source: retrospective canine leishmaniasis database, Centre National de Référence des Leishmania.
**Istituto Superiore di Sanità (ISS), Rome, Italy.
††Hellenic Pasteur Institute (HPI), Athens, Greece.
‡‡National Reference Laboratory for Animal Health (VS), Nicosia, Cyprus.
§§Ege University (EUMS-DP), Izmir, Turkey.

(17) had been infected within European Union boundaries: Spain, Portugal, Greece, or France (*10*). Five cases were in children. Similarly, a retrospective study in the Hospital for Tropical Diseases in London showed that most of the imported VL case-patients in the United Kingdom were adult men touring the Mediterranean (*11*). Second, in the absence of public health surveillance at the European level, underreporting is common (see the Leishmaniasis and the Globalization of Neglect section). Third, asymptomatic infections may be common in some regions: for 1 clinical case of VL, there may be 30–100 subclinical infections (*12*). This underreporting can have major consequences for blood banks: blood from donors living in areas of endemicity in southern France and Greece had 3.4% and 15% seropositivity, respectively (*13*,*14*). In addition, 22.1% of blood donors in a highly disease-endemic area from Spain were PCR positive for leishmaniasis (*15*). Furthermore, asymptomatic infections may progress to severe clinical forms in immunocompromised persons, for example, in AIDS patients (*16*). Fourth, the etiologic agent of southern European VL, *Leishmania infantum*, is also infecting dogs (with a seroprevalence of up to 34% in areas of Spain where the disease is highly endemic) (Table). Dogs with leishmaniasis infections are generally very sick, causing a major problem in southern Europe (e.g., ≈5,000 clinical cases occur each year in France) (Table). However, sick as well as asymptomatic dogs also represent a risk for humans, as they constitute the major reservoir of the parasite on which sand fly vectors may feed and transmit the infection.

Import–Export Balance of European Leishmaniasis

In addition to the reality of autochthonous leishmaniasis in Europe, the risk for introduction of new species through travelers or immigrants from countries where

non-European species are endemic should also be considered. However, the probability that these species could enter in a transmission cycle is relatively low. The probability depends on contact between infected persons and sand flies, the capacity of the infected person to act as reservoir, and the susceptibility of European sand flies to the different *Leishmania* species. For most species, humans are generally a transmission dead-end. However, for 2 species, the risk might theoretically be higher: *L. tropica*, which is causing CL in Africa, the Middle East, and Southwest Asia, and *L. donovani*, the etiologic agent of VL in East Africa and the Indian subcontinent. These 2 species are indeed associated with an anthroponotic transmission cycle. On one hand, *L. donovani*, which is transmitted by a different species of sand fly outside Europe, might be hosted by most European sand flies, except *Phlebotomus papatasi* and *P. sergenti* (*17*). On the other hand, *L. tropica*, which has more stringent requirements in terms of vector, would need *P. sergenti*, which was reported in several places in southern Europe, from Portugal (*18*) to Cyprus (*19*). *L. tropica* was indeed encountered in Greece (*20*), and according to a very recent report, the first autochthonous cases of *L. donovani* in Europe have been detected in Cyprus (*21*). The clinical phenotype associated with both species needs also to be considered for an exhaustive risk evaluation. *L. tropica* causes lesions that are generally more difficult to treat with antimonial drugs (*22*), whereas *L. donovani* is considered to be more aggressive than *L. infantum* and often does not respond to treatment with first-line drugs (*23*).

In addition to being concerned about importation and spread of exotic *Leishmania* species in Europe, exportation should also be considered. The best known historical example of the spread of leishmaniasis is the migration of *L. infantum* from Europe to Latin America, where it colonized in *Lutzomyia longipalpis* and is now causing a serious public health problem (>3,500 cases of VL per year in Brazil) (*24*). This spread is thought to have been caused by conquistadores' dogs (*25*). Another and current example concerns the *L. major/L. infantum* hybrids recently described in HIV-positive VL patients from Portugal (*26*). Indeed, these hybrids were shown to be able to develop in *P. papatasi* (*27*), a vector that is widespread in Europe, Africa, and Asia. Considering the reservoir role of HIV–coinfected patients and the peridomestic and anthropophilic nature of *P. papatasi*, these hybrid strains might circulate by using this sand fly vector, thereby increasing the risk of their spreading into new foci throughout the broad range of *P. papatasi* distribution (*27*). Finally, the way Europe deals with its leishmaniasis public and animal health problem can still have major consequences for the rest of the world. Miltefosine, one of the few available antileishmania drugs, has been recently launched in the market for canine leishmaniasis treatment in Portugal, Spain, Italy, Greece, and Cyprus. Because dogs are never cured parasitologically and given the long half-life of the drug, the lack of European policy might contribute to the emergence of parasites resistant to miltefosine. This resistance could be a problem for European human patients, as miltefosine is being used on a compassionate basis in several European AIDS coinfected patients unresponsive to amphotericin B or pentavalent antimonials (*28*,*29*). Furthermore, if dogs infected with miltefosine-resistant strains were to migrate to Latin America, where several countries have registered the drug for human use (currently Colombia, Guatemala, Argentina, Venezuela, Paraguay, Ecuador, and Honduras; *30*), the impact might be greater.

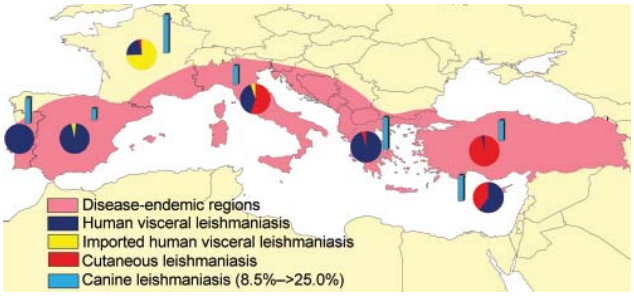


Figure. Leishmaniasis in southern Europe. Distribution of the endemic disease; relative proportion of autochthonous (visceral, cutaneous) and imported human cases and seroprevalence in dogs (from data reported in Table).

Leishmaniasis and the Globalization of Neglect

Twelve million persons have leishmaniasis, and 500,000 new cases of VL occur each year. More than 50,000 die of this disease each year. The disease is spreading because of several risk factors, climate being only one. Humanmade changes to the environment and population movements (for economic or political reasons) may lead to alterations in the range and densities of the vectors and reservoirs, increasing human exposure to infected sand flies. Urbanization of leishmaniasis becomes more common and in conjunction with the ruralization of HIV/AIDS, it contributes to increase the problem of co-infections in contexts where access to highly active antiretroviral therapy is not the same as in industrialized countries. *Leishmania* spp. have already become resistant to antimonial drugs (the first-line drug in many developing countries) in some regions and may soon become resistant to miltefosine (*23*). Despite this increasing resistance, leishmaniasis is one of the most neglected diseases in developing countries, along with others like sleeping sickness or Chagas disease. Leishmaniasis is a disease for which we lack effective, affordable, and easy to use drugs, and the pharmaceutical industry has had few incentives to engage

in their development. In addition, leishmaniasis surveillance and control are also neglected. One of the main reasons for this neglect is that in developing countries, leishmaniasis is a disease of the poor. Risk for infection and clinical development are mediated by poverty, while leishmaniasis diagnosis and treatment are expensive and may lead to further impoverishment and reinforcement of the vicious cycle of disease and poverty (*31*).

In Europe, physicians are sometimes ill-informed on the diagnosis and treatment of leishmaniasis. In France, a telephone advice line was created in 2006 by the National Reference Centre of Leishmania to help physicians in their therapeutic diagnosis. A study in Germany, a non–disease-endemic country, showed that the median time between symptom onset and correct diagnosis was 85 and 61 days in case-patients of VL and CL, respectively (*32*). This value was lower in a leishmaniasis-endemic area, such as southern Italy (35 days, [*33*]). VL, which was initially a pediatric disease in Europe (hence the name of *L. infantum*), only began to gain attention when the co-infection of HIV/AIDS was documented. Between the late 1980s and early 2001, >1,900 cases were reported in southwestern Europe (*16*). Even though it was reported that both pathogens could be transmitted through sharing of needles among intravenous drug users (*34*), in many cases of co-infection, the parasite was already present at the time of HIV infection, which indicates that HIV infection would have an unmasking effect on the true endemicity of *Leishmania* infection. In other words, the wave of *Leishmania*/HIV co-infection showed that *L. infantum* could behave as an opportunistic parasite, with many asymptomatic carriers (*12*), and with the clinical syndromes being only the tip of the iceberg. Because of the highly active antiretroviral therapy, cases of co-infection generally decreased in the region, with the exception of Portugal (*35*).

Notification of VL varies according to the country. It does not belong to the list of 30 notifiable diseases in France. However, notification is compulsory in Greece, Italy, and Portugal, though only obligatory in 12 of 17 autonomous communities of Spain. Underreporting is common. In Portugal, for instance, 76 cases of autochthonous VL were officially reported at the country level from 2000 through 2005. During the same period, 127 cases (+67%) were observed in the Institute of Tropical Medicine of Lisboa (Table). In the case of autochthonous cutaneous leishmaniasis, consolidated data are lacking, but this clinical form is definitely underreported because of its benign nature and the fact that it usually does not require hospitalization. Nonetheless, leishmaniasis is not a disease placed under public health surveillance at the European level. It does not even belong to the package of rare diseases considered as a priority in the Public Health Programme 2003–2008. (Rare diseases, including those of genetic origin, are life-threatening or chronically debilitating diseases that are of such low prevalence [<5/10,000 persons] that special combined efforts are needed to address them so as to prevent significant illness or perinatal or early deaths or a considerable reduction in a person's quality of life or socioeconomic potential.) At the regional level, the only dedicated network of surveillance was the one launched by the World Health Organization and the Joint United Nations Programme on HIV/AIDS in 1993 for the surveillance of *Leishmania*/HIV co-infections, which essentially involved European countries as well as some developing countries.

The low-profile perception seen for human leishmaniasis differs dramatically from the veterinary world's perception. The high incidence of canine leishmaniasis in southern Europe makes *Leishmania* one of the main dog killers in the region, and private veterinarians are well aware of it. Dogs are treated individually to protect from sand fly bites, and those diagnosed as infected are considered extremely difficult to treat. Specific web sites are available for owners of infected dogs to discuss and compare treatment regimens and pose questions to veterinarians. Several pharmaceutical companies are investing in research and development of vaccines, drugs, and topical insecticides for specific cure and prevention of canine leishmaniasis. This high-profile perception, however, drops when dogs must be treated as the reservoir of human leishmaniasis. For instance, the issue of notification is treated differently in various leishmaniasis–endemic countries, but even where notification is compulsory (i.e., Italy and Spain), it is not a common practice. In Italy, the network Leishmap is currently monitoring the spread of canine leishmaniasis and vectors in northern Italy. Leishmap is a scientific network, supported by a private company (*36*). Furthermore, private interests are sometimes at odds with public health goals. Drugs for leishmaniasis are not regulated in the veterinary market, and medications intended for use in humans, such as Ambisome, are used in domestic pets, with the potential risk that they might be a source for the emergence and spreading of resistant strains.

Countering the Neglect

Since 2001, several research consortia gathered scientists from Euro-Mediterranean countries (www.leishrisk.net). These consortia and other research groups generated knowledge, tools, and education packages and led to a solid European research network dedicated to the study of leishmaniasis. Bridging research with surveillance and control is an issue of dialogue and advocacy. On one hand, health professionals need to be in close contact with scientists to help translate basic research into relevant and applicable tools. For instance, sequencing the whole genome of *Leishmania* represented a technologic challenge, but the next challenge is to exploit this sequencing for the benefit of the patient (www.leishrisk.net). On the other hand, scientists

must market their results to influence health policy. Changes in health policy are being made; during manuscript revision, we were informed of the selection of leishmaniasis among the priority zoonoses addressed by the Episouth network (www.leishrisk.net).

Deciding health policy is a complex social, economic, and political interrelationship that is much broader than leishmaniasis alone (or even infectious diseases generally). However, if Europe justifiably wants to invest more in surveillance of vector-borne diseases, the time has come to recognize its real impact on both animal and human health and include leishmaniasis as one of these diseases.

Acknowledgments

We thank Kirami Olgen for the preparation of the map on leishmaniasis.

LEISHMED (coordination action) and LEISHRISK (specific support action) are funded by the 6th Frame Programme of European Union (Programme INCO): contracts INCO-CT-2004-509086 and INCO 043490, respectively.

Dr Dujardin is the head of the Molecular Parasitology Unit at the Institute of Tropical Medicine of Antwerp, Belgium. He specializes in the molecular epidemiology of leishmaniasis and in natural drug resistance.

References

1. Watson R. Europe witnesses first local transmission of chikungunya fever in Italy. BMJ. 2007;335:532–3. DOI: 10.1136/bmj.39332.708738.DB
2. Lines J. Chikungunya in Italy. Globalisation is to blame, not climate change. BMJ. 2007;335:576. DOI: 10.1136/bmj.39342.563310.80
3. Cortes S, Afonso MO, Alves-Pires C, Campino L. Stray dogs and leishmaniasis in urban areas, Portugal. Emerg Infect Dis. 2007;13:1431–2.
4. Alvar J. Las leishmaniasis: de la biología al control, 2001. Salamanca: Laboratorios Intervet S.A.; 2001.
5. Miró G, Molina R. Leishmaniosis canina: Manejo clínico y situación actual en España, 2006. Spain: Bayer Health Care; 2006
6. Gradoni L. Epizootiology of canine leishmaniasis in southern Europe. In: R. Killick-Kendrick, editor. Canine leishmaniasis: an update. Wiesbaden (Germany): Hoechst Roussel Vet; 1999. p. 32–9.
7. Papadopoulou C, Kostoula A, Dimitriou D, Panagiou A, Bobojianni C, Antoniades GJ. Human and canine leishmaniasis in asymptomatic and symptomatic population in Northwestern Greece. J Infect. 2005;50:53–60. DOI: 10.1016/j.jinf.2004.05.004
8. Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, et al. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop Med Int Health. 2008;13:256–64.
9. Bogdan C, Schonian G, Banuls AL, Hide M, Pratlong F, Lorenz E, et al. Visceral leishmaniasis in a German child who had never entered a known endemic area: case report and review of the literature. Clin Infect Dis. 2001;32:302–6. DOI: 10.1086/318476
10. Harms G, Schönian G, Feldmeier H. Leishmaniasis in Germany. Emerg Infect Dis. 2003;9:872–5.
11. Malik ANJ, John L, Bryceson ADM, Lockwood DNJ. Changing pattern of visceral leishmaniasis, United Kingdom, 1985–2004. Emerg Infect Dis. 2006;12:1257–9.
12. Pampiglione S, Manson-Bahr PE, La Placa M, Borgatti MA, Musumeci S. Studies in Mediterranean leishmaniasis. 3. The leishmanin skin test in kala-azar. Trans R Soc Trop Med Hyg. 1975;69:60–8. DOI: 10.1016/0035-9203(75)90012-7
13. Le Fichoux Y, Quaranta JF, Aufeuvre JP, Lelievre A, Marty P, Suffia I, et al. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. J Clin Microbiol. 1999;37:1953–7.
14. Kyriakou DS, Alexandrakis MG, Passam FH, Kourelis TV, Foundouli P, Matalliotakis E, et al. Quick detection of *Leishmania* in peripheral blood by flow cytometry. Is prestorage leucodepletion necessary for leishmaniasis prevention in endemic areas? Transfus Med. 2003;13:59–62. DOI: 10.1046/j.1365-3148.2003.00420.x
15. Riera C, Fisa R, Udina M, Gállego M, Portus M. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans R Soc Trop Med Hyg. 2004;98:102–10. DOI: 10.1016/S0035-9203(03)00015-4
16. Desjeux P, Alvar J. *Leishmania*/HIV co-infections: epidemiology in Europe. Ann Trop Med Parasitol. 2003;97(Suppl 1):3–15. DOI: 10.1179/000349803225002499
17. Myskova J, Svobodova M, Beverley SM, Volf P. A lipophosphoglycan-independent development of *Leishmania* in permissive sand flies. Microbes Infect. 2007;9:317–24. DOI: 10.1016/j.micinf.2006.12.010
18. Afonso MO, Campino L, Cortes S, Alves-Pires C. The phlebotomine sandflies of Portugal. XIII–Occurrence of *Phlebotomus sergenti* Parrot, 1917 in the Arrabida leishmaniasis focus. Parasite. 2005;12:69–72.
19. Depaquit J, Léger N, Ferté H, Rioux JA, Gantier JC, Michaelides A, et al. Phlebotomines of the Isle of Cyprus. III. Species inventory. Parasite. 2001;8:11–20.
20. Garifallou A, Schnur LF, Stratigos JD, Hadziandoniou M, Savigos M, Stavrianeas N, et al. Leishmaniasis in Greece II. Isolation and identification of the parasite causing cutaneous leishmaniasis in man. Ann Trop Med Parasitol. 1984;78:369–75.
21. Antoniou M, Haralambous C, Mazeris A, Pratlong F, Dedet J-P, Soteriadou K. *Leishmania donovani* leishmaniasis in Cyprus. Lancet Infect Dis. 2008;8:6–7. DOI: 10.1016/S1473-3099(07)70297-9
22. Hadighi R, Mohebali M, Boucher P, Hajjaran H, Khamesipour A, Ouellette M. Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. PLoS Med. 2006;3:e162. DOI: 10.1371/journal.pmed.0030162
23. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. Clin Microbiol Rev. 2006;19:111–26. DOI: 10.1128/CMR.19.1.111-126.2006
24. Miles MA, Vexenat JA, Furtado Campos JH, Fonseca de Castro JA. Canine leishmaniasis in Latin America: control strategies for visceral leishmaniasis. In: R. Killick-Kendrick, editor. Canine leishmaniasis: an update. Barcelona: Hoechst Roussel Vet;1999. p. 46–53.
25. Mauricio IL, Stothard JR, Miles MA. The strange case of *Leishmania chagasi*. Parasitol Today. 2000;16:188–9. DOI: 10.1016/S0169-4758(00)01637-9
26. Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, Campino L. First report of genetic hybrids between two very divergent *Leishmania* species: *Leishmania infantum* and *Leishmania major.* Int J Parasitol. 2006;36:1383–8. DOI: 10.1016/j.ijpara.2006.06.019
27. Volf P, Benkova I, Myskova J, Sadlova J, Campino L, Ravel C. Increased transmission potential of *Leishmania major*/*Leishmania infantum* hybrids. Int J Parasitol. 2007;37:589–93. Epub 2007 Feb 15. DOI: 10.1016/j.ijpara.2007.02.002
28. Schraner C, Hasse B, Hasse U, Baumann D, Faeh A, Burg G, et al. Successful treatment with miltefosine of disseminated cutaneous leishmaniasis in a severely immunocompromised patient infected with HIV-1. Clin Infect Dis. 2005;40:e120–4. DOI: 10.1086/430444

29. Troya J, Casquero A, Refoyo E, Fernández-Guerrero ML, Górgolas M. Long term failure of miltefosine in the treatment of refractory visceral leishmaniasis in AIDS patients. Scand J Infect Dis. 2008;40:78–80. DOI: 10.1080/00365540701466215
30. World Health Organization. Eighteenth programme report/progress 2005–2006. Geneva: The Organization; 2007.
31. Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. Trends Parasitol. 2006;22:552–7. DOI: 10.1016/j.pt.2006.09.004
32. Weitzel T, Mühlberger N, Jelinek T, Schunk M, Ehrhardt S, Bogdan C, et al. Imported leishmaniasis in Germany 2001–2004: data of the SIMPID surveillance network [in German]. Eur J Clin Microbiol Infect Dis. 2005;24:471–6. DOI: 10.1007/s10096-005-1363-1
33. Pagliano P, Rossi M, Rescigno C, Altieri S, Coppola MG, Gramiccia M, et al. Mediterranean visceral leishmaniasis in HIV-negative adults: a retrospective analysis of 64 consecutive cases (1995–2001). J Antimicrob Chemother. 2003;52:264–8. DOI: 10.1093/jac/dkg340
34. Alvar J, Jimenez M. Could infected drug-users be potential *Leishmania infantum* reservoirs? AIDS. 1994;8:854. DOI: 10.1097/00002030-199406000-00024
35. World Health Organization. Report of the fifth Consultative Meeting on HIV-*Leishmania* Co-Infection, Addis Ababa 20–22 March 2007. Geneva: The Organization; 2007.
36. Capelli G, Baldelli R, Ferroglio E, Genchi C, Gradoni L, Gramiccia M, et al. Monitoring of canine leishmaniasis in northern Italy: an update from a scientific network. Parassitologia. 2004;46:193–7.

Address for correspondence: Jean-Claude Dujardin, Instituut voor Tropische Geneeskunde, Molecular Parasitology, Nationalestraat 155, B-2000 Antwerp, Belgium; email: jcdujardin@itg.be

Rickettsia felis as Emergent Global Threat for Humans

Carlos E. Pérez-Osorio,* Jorge E. Zavala-Velázquez,* Juan José Arias León,* and Jorge E. Zavala-Castro*

Rickettsia felis is an emergent pathogen belonging to transitional group rickettsiae. First described in 1990, *R. felis* infections have been reported to occur worldwide in fleas, mammals, and humans. Because clinical signs of the illness are similar to those of murine typhus and other febrile illnesses such as dengue, the infection in humans is likely underestimated. *R. felis* has been found throughout the world in several types of ectoparasites; cat fleas appear to be the most common vectors. *R. felis* infection should be considered an emergent threat to human health.

Rickettsia felis is a member of the genus *Rickettsia*, which comprises intracellular pathogens that produce infections commonly called rickettsioses. Although the genus has no recognized subspecies, rickettsiae have traditionally been subdivided into 2 groups: the spotted fever group (SFG) and the typhus group. Infections produced by these 2 groups are clinically indistinguishable; however, groups can be differentiated by outer membrane protein OmpA (absent in the typhus group) and by vector. SFG members are transmitted by ticks; typhus group members, by fleas and lice (*1*,*2*). More recently, Gillespie et al. (*3*) added to this classification by designating the transitional group of rickettsiae and describing an ancestral group of rickettsiae.

In 1990, Adams et al. described a rickettsia-like organism, which resembled *R. typhi*, in the cytoplasm of midgut cells of a colony of cat fleas (*1*). The new rickettsia received the initial name of ELB agent after the company from which the fleas were obtained (El Labs, Soquel, CA, USA) (*4*). The first observations, such as reactivity with antibodies to *R. typhi* (*1*), the type of vector in which it was first discovered (*1*), and the apparent absence of OmpA (*5*), suggested that the new organism belonged to the typhus group of rickettsiae (*4*).

The molecular characterization of the organism described by Adams and reported by Bouyer et al. in 2001 provided sufficient evidence to support the designation of *R. felis* as a member of the SFG (*6*), and in 2002, La Scola et al. provided further characterization (*7*). One noticeable characteristic is the temperature-dependent growth of the bacterium, which requires incubation temperatures of 28°–32°C for optimal growth. However, the most striking characteristic of the novel rickettsia was the plasmid DNA in its genome (*8*).

World Distribution in Potential Host Vectors

Soon after the initial description of the typhus-like rickettsia, Williams et al. (*9*) reported that cat fleas collected from opossums in an urban setting in California were infected with the novel rickettsia, but no organism was detected in the tissues of the opossums. Since this report, this organism has been described in infected vectors from 20 countries on 5 continents (*9*). Not until 2002 did interest in *R. felis* increase, when the United States (*9*), Brazil (*10*), Mexico (*11*), and Spain (*12*) were among the first countries to describe cat fleas (*Ctenocephalides felis*) infected with *R. felis.* During the following 5 years, 28 additional reports appeared from all over the world (Table 1). These reports describe new potential vectors being infected with the emergent rickettsia, including the following: fleas, such as *C. canis* (*13–15*), *Anomiopsyllus nudata* (*16*), *Archaeopsylla erinacei* (*15*,*17*), *Ctenophthalmus* sp.

*Universidad Autónoma de Yucatán, Mérida, México

DOI: 10.3201/eid1407.071656

Table 1. Potential vectors infected with *Rickettsia felis* reported worldwide, 1992–2007*

Year	Source of DNA sample	Animal†	Country	Reference
1992	*Ctenocephalides felis*	Opossum	USA	(*9*)
2002	*C. felis*	Cats and dogs	Brazil	(*10*)
2002	*C. felis*	Dogs	Mexico	(*11*)
2002	*C.felis*	Cats and dogs	Spain	(*12*)
2003	*Haemophysalis flava, H. kitaokai,* and *Ixodes ovatus*	Unknown (flagging)	Japan	(*19*)
2003	*C. felis*	Cats	France	(*22*)
2003	*C. felis*	Cats and dogs	UK	(*23*)
2004	*C. felis*	Dogs	Peru	(*24*)
2005	*Anomiopsyllus nudata*	Wild rodents	USA	(*16*)
2005	*C. felis*	Cats and dogs	New Zealand	(*25*)
2005	*C. felis*	Monkey	Gabon	(*26*)
2006	*C. felis* and *C. canis*	Dogs	Brazil	(*13*)
2006	*C. felis* and *C. canis*	Cats and dogs	Uruguay	(*14*)
2006	*Archaeopsylla erinacei* and *C. canis*	Hedgehog and rodents	Algeria	(*15*)
2006	*A. erinacei* and *Ctenophtalmus sp.*	Rodents and hedgehog	Portugal	(*17*)
2006	*Xenopsylla cheopis*	Rodents‡	Indonesia	(*18*)
2006	*C. felis, Rhipicephalus sanguineus,* and *Amblyommma cajennense*	Dogs and horse	Brazil	(*20*)
2006	Unknown flea	Gerbil	Afghanistan	(*27*)
2006	*C. felis*	Cats and dogs	Australia	(*28*)
2006	*C. felis*	Cats	Israel	(*29*)
2006	*C. felis*	Rodents	Cyprus	(*30*)
2007	Mites	Wild rodents	South Korea	(*21*)
2007	*C. felis*	Cats	USA	(*31*)
2007	*C. felis*	Cats	Chile	(*32*)

*PCR was used to detect *R. felis* infection with 1 noted exception.
†Animal host of potential vectors.
‡Quantitative PCR.

(*17*), and *Xenopsylla cheopis* (*18*); ticks, *Haemaphysalis flava* (*19*), *Rhipicephalus sanguineus* (*20*), and *Ixodes ovatus* (*19*); and mites from South Korea (*21*) (Table 1). Despite the large number of potential vectors reported, the only vector currently recognized is *C. felis* because it has been demonstrated that this flea is able to maintain a stable infected progeny through transovarial transmission (*4*). In addition, production of antibody to *R. felis* has been noted in animals after they have been exposed to infected cat fleas (*9*). Other evidence to be considered is the fact that 68.8% of the reports state that the cat flea is the most recurrent vector in which *R. felis* has been detected. These data further support the wide distribution of rickettsiae because they correlate with the worldwide distribution of *C. felis;* this distribution represents a threat to the human population because of lack of host specificity of the cat flea.

R. felis infection is diagnosed by PCR amplification of targeted genes. The genes most commonly amplified by researchers are *gltA* and *ompB*; followed by the 17-kDa gene. Also, 25% of published articles report that *R. felis* was detected by amplifying >2 genes, and all report that amplicons were confirmed as *R. felis* by sequencing. The animal hosts from which the infected ectoparasites were recovered represent a diversity of mammals (Table 1), which included 9 different naturally infested animal species. However, in 16 of 33 articles, ectoparasites were recovered from dogs. Other hosts for ectoparasites were cats (in 13 of 33 reports); rodents (5 of 33 reports); opossums and hedgehogs (2 reports each); and horses, sheep, goats, gerbils, and monkeys (1 report for each animal species).

In summary, the presence of *R. felis* in a diverse range of invertebrate and mammalian hosts represents a high potential risk for public health and the need for further studies to establish the role of ectoparasites other than *C. felis* as potential vectors. To date, whether any vertebrate may serve as the reservoir of this emergent pathogen has not been determined. However, preliminary data from our laboratory suggest that opossums are the most likely candidates.

World Distribution of Human Cases

In 1994, the first human case of infection with the new cat flea rickettsia was reported in the United States (*2*). This became the first evidence of *R. felis'* potential as a human pathogen. *R. felis* infection had a similar clinical manifestation as murine typhus (including high fever [39°–40°C], myalgia, and rash). Although the initial idea was that the murine typhus–like rickettsia had a transmission cycle involving cat fleas and opossums (*2*,*5*,*9*), no viable *R. felis* has yet been isolated from a vertebrate host.

Three more cases of *R. felis* infection were reported from southeastern Mexico in 2000. The patients had had contact with fleas or animals known to carry fleas. The clinical manifestations were those of a typical rickettsiosis: all patients had fever and myalgia; but the skin lesions, instead of a rash, were similar to those described for rickettsialpox. In addition, for 3 patients, central nervous system involvement developed, manifested as photophobia, hearing loss, and signs of meningitis (*33*).

As occurred with the fast-growing reports of the worldwide detection of *R. felis* in arthropod hosts, the reports of human cases of *R. felis* infection increased rapidly in the following years (Table 2). But, in contrast, only 11 articles reported human infection by *R. felis* compared with 32 that reported ectoparasite infection with the new rickettsia. Nevertheless, these findings indicate that an effective surveillance system is urgently needed to distinguish *R. felis* rickettsiosis from other rickettsial infections such as murine typhus and Rocky Mountain spotted fever, and from other febrile illnesses such as dengue. Although PCR is still a method of choice for many laboratories, its high cost prevents many from using the technique, particularly in developing countries. Important advances have been achieved in diagnostics, such as the recent establishment of a stable culture of *R. felis* in cell lines that allows its use as antigen in serologic assays differentiating the cat flea rickettsia from others. Use of this culture in the immunofluorecent assay has enabled detection of additional human cases (*38*).

The first autochthonous human case in Europe was reported in 2002, which demonstrated that *R. felis* has a potential widespread distribution and is not confined to the Americas. It also confirmed the risk for human disease anywhere in the world. After the first report in Europe of a human infection of *R. felis*, other human cases have appeared in other countries around the world, including Thailand (*36*), Tunisia (*38*), Laos (*39*), and Spain (*40*); additional cases have been reported in Mexico and Brazil (*34*). All the data support the conclusion that the incidence of *R. felis* rickettsiosis and the simultaneous worldwide distribution of the flea vector plausibly explain its endemicity.

At present, the involvement of domestic animals (e.g., dogs and cats) or wild animals coexisting in urban areas (e.g., opossums) maintains *R. felis* infection in nature. *C. felis* fleas serve as the main reservoir and likely have a central role in transmission of human illness.

Conclusions

R. felis is an emergent rickettsial pathogen with a worldwide distribution in mammals, humans, and ectoparasites. The clinical manifestations of *R. felis* infections resemble those of murine typhus and dengue, which makes them difficult to diagnose without an appropriate laboratory test. For this reason, infections due to this emergent pathogen are likely underestimated and misdiagnosed. Although *R. felis* may require only fleas for its maintenance in nature, we still do not know the role of animals in the life cycle of flea-borne spotted fever rickettsia. In addition, flea-borne spotted fever should be considered in the differential diagnosis of infectious diseases. Further research should be conducted to determine the actual incidence of *R. felis* infection in humans, the spectrum of clinical signs and symptoms, and the severity of this infection and also to assess the impact on public health.

Acknowledgment

We thank Patricia Croquet-Valdes for her comments and helpful advice.

This research was supported by grants from the CONACyT (44064-M) to J. E. Z.-V.

Mr. Perez-Osorio is currently professor of microbiology at the Autonomous University of Yucatan. His research interests focus on rickettsial diseases.

References

1. Adams JR, Schmidtmann ET, Azad AF. Infection of colonized cat fleas, *Ctenocephalides felis* (Bouche), with a rickettsia-like microorganism. Am J Trop Med Hyg. 1990;43:400–9.
2. Schriefer ME, Sacci JB Jr, Dumler JS, Bullen MG, Azad AF. Identification of a novel rickettsial infection in a patient diagnosed with murine typhus. J Clin Microbiol. 1994;32:949–54.

Table 2. Human cases of *Rickettsis felis* infection reported worldwide, 1994–2006*

Year	No. cases	Method	Country	Reference
1994	1	PCR	USA	(*2*)
2000, 2006	5	PCR	Mexico	(*33*)
2001, 2006	3	PCR	Brazil	(*34*)
2002	2	PCR/serology	Germany	(*35*)
2003	1	Serology (seroconversion)	Thailand	(*36*)
2005	3	Serology (Western blot)	South Korea	(*37*)
2006	8	Serology (IFAT/Western blot)	Tunisia	(*38*)
2006	1	Serology (seroconversion)	Laos	(*39*)
2006	33	Serology (IFAT)	Spain	(*40*)
Total	68			

*IFAT, indirect fluorescent antibody test.

3. Gillespie JJ, Beier MS, Rahman MS, Ammerman NC, Shallom JM, Purkayastha A, et al. Plasmids and rickettsial evolution: insight from *Rickettsia felis*. PLoS One. 2007;2:e266. DOI: 10.1371/journal.pone.0000266
4. Azad AF, Sacci JB Jr, Nelson WM, Dasch GA, Schmidtmann ET, Carl M. Genetic characterization and transovarial transmission of a typhus-like rickettsia found in cat fleas. Proc Natl Acad Sci U S A. 1992;89:43–6. DOI: 10.1073/pnas.89.1.43
5. Higgins JA, Radulovic S, Schriefer ME, Azad AF. *Rickettsia felis*: a new species of pathogenic rickettsia isolated from cat fleas. J Clin Microbiol. 1996;34:671–4.
6. Bouyer DH, Stenos J, Crocquet-Valdes P, Moron CG, Popov VL, Zavala-Velazquez JE, et al. *Rickettsia felis*: molecular characterization of a new member of the spotted fever group. Int J Syst Evol Microbiol. 2001;51:339–47.
7. La Scola B, Meconi S, Fenollar F, Rolain JM, Roux V, Raoult D. Emended description of *Rickettsia felis* (Bouyer et al. 2001), a temperature-dependent cultured bacterium. Int J Syst Evol Microbiol. 2002;52:2035–41. DOI: 10.1099/ijs.0.02070-0
8. Ogata H, Renesto P, Audic S, Robert C, Blanc G, Fournier PE, et al. The genome sequence of *Rickettsia felis* identifies the first putative conjugative plasmid in an obligate intracellular parasite. PLoS Biol. 2005;3:e248. DOI: 10.1371/journal.pbio.0030248
9. Williams SG, Sacci JB Jr, Schriefer ME, Andersen EM, Fujioka KK, Sorvillo FJ, et al. Typhus and typhuslike rickettsiae associated with opossums and their fleas in Los Angeles County, California. J Clin Microbiol. 1992;30:1758–62.
10. Oliveira RP, Galvao MA, Mafra CL, Chamone CB, Calic SB, Silva SU, et al. *Rickettsia felis* in *Ctenocephalides* spp. fleas, Brazil. Emerg Infect Dis. 2002;8:317–9.
11. Zavala-Velazquez JE, Zavala-Castro JE, Vado-Solis I, Ruiz-Sosa JA, Moron CG, Bouyer DH, et al. Identification of *Ctenocephalides felis* fleas as a host of *Rickettsia felis*, the agent of a spotted fever rickettsiosis in Yucatan, Mexico. Vector Borne Zoonotic Dis. 2002;2:69–75. DOI: 10.1089/153036602321131869
12. Marquez FJ, Muniain MA, Perez JM, Pachon J. Presence of *Rickettsia felis* in the cat flea from southwestern Europe. Emerg Infect Dis. 2002;8:89–91.
13. Horta MC, Chiebao DP, de Souza DB, Ferreira F, Pinheiro SR, Labruna MB, et al. Prevalence of *Rickettsia felis* in the fleas *Ctenocephalides felis* and *Ctenocephalides canis* from two Indian villages in São Paulo Municipality, Brazil. Ann N Y Acad Sci. 2006;1078:361–3. DOI: 10.1196/annals.1374.071
14. Venzal JM, Perez-Martinez L, Felix ML, Portillo A, Blanco JR, Oteo JA. Prevalence of *Rickettsia felis* in *Ctenocephalides felis* and *Ctenocephalides canis* from Uruguay. Ann N Y Acad Sci. 2006;1078:305–8. DOI: 10.1196/annals.1374.056
15. Bitam I, Parola P, De La Cruz KD, 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.
16. Stevenson HL, Labruna MB, Montenieri JA, Kosoy MY, Gage KL, Walker DH. Detection of *Rickettsia felis* in a New World flea species, *Anomiopsyllus nudata* (*Siphonaptera: Ctenophthalmidae*). J Med Entomol. 2005;42:163–7. DOI: 10.1603/0022-2585-(2005)042[0163:DORFIA]2.0.CO;2
17. De Sousa R, Edouard-Fournier P, Santos-Silva M, Amaro F, Bacellar F, Raoult D. Molecular detection of *Rickettsia felis*, *Rickettsia typhi* and two genotypes closely related to *Bartonella elizabethae*. Am J Trop Med Hyg. 2006;75:727–31.
18. Jiang J, Soeatmadji DW, Henry KM, Ratiwayanto S, Bangs MJ, Richards AL. *Rickettsia felis* in *Xenopsylla cheopis*, Java, Indonesia. Emerg Infect Dis. 2006;12:1281–3.
19. Ishikura M, Ando S, Shinagawa Y, Matsuura K, Hasegawa S, Nakayama T, et al. Phylogenetic analysis of spotted fever group rickettsiae based on *gltA*, 17-kDa, and rOmpA genes amplified by nested PCR from ticks in Japan. Microbiol Immunol. 2003;47:823–32.
20. Cardoso LD, Freitas RN, Mafra CL, Neves CV, Figueira FC, Labruna MB, et al. Characterization of *Rickettsia* spp. circulating in a silent peri-urban focus for Brazilian spotted fever in Caratinga, Minas Gerais, Brazil [in Portuguese]. Cad Saude Publica. 2006;22:495–501.
21. Choi YJ, Lee EM, Park JM, Lee KM, Han SH, Kim JK, et al. Molecular detection of various rickettsiae in mites (Acari: Trombiculidae) in southern Jeolla Province, Korea. Microbiol Immunol. 2007;51:307–12.
22. 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.
23. Kenny MJ, Birtles RJ, Day MJ, Shaw SE. *Rickettsia felis* in the United Kingdom. Emerg Infect Dis. 2003;9:1023–4.
24. Blair PJ, Jiang J, Schoeler GB, Moron C, Anaya E, Cespedes M, et al. Characterization of spotted fever group rickettsiae in flea and tick specimens from northern Peru. J Clin Microbiol. 2004;42:4961–7. DOI: 10.1128/JCM.42.11.4961-4967.2004
25. Kelly P, Rolain JM, Raoult D. Prevalence of human pathogens in cat and dog fleas in New Zealand. N Z Med J. 2005;118:U1754.
26. Rolain JM, Bourry O, Davoust B, Raoult D. *Bartonella quintana* and *Rickettsia felis* in Gabon. Emerg Infect Dis. 2005;11:1742–4.
27. Marie JL, Fournier PE, Rolain JM, Briolant S, Davoust B, Raoult D. Molecular detection of *Bartonella quintana, B. elizabethae, B. koehlerae, B. doshiae, B. taylorii*, and *Rickettsia felis* in rodent fleas collected in Kabul, Afghanistan. Am J Trop Med Hyg. 2006;74:436–9.
28. Schloderer D, Owen H, Clark P, Stenos J, Fenwick SG. *Rickettsia felis* in fleas, Western Australia. Emerg Infect Dis. 2006;12:841–3.
29. Bauer O, Baneth G, Eshkol T, Shaw SE, Harrus S. Polygenic detection of *Rickettsia felis* in cat fleas (*Ctenocephalides felis*) from Israel. Am J Trop Med Hyg. 2006;74:444–8.
30. Psaroulaki A, Antoniou M, Papaeustathiou A, Toumazos P, Loukaides F, Tselentis Y. First detection of *Rickettsia felis* in *Ctenocephalides felis* fleas parasitizing rats in Cyprus. Am J Trop Med Hyg. 2006;74:120–2.
31. Hawley JR, Shaw SE, Lappin MR. Prevalence of *Rickettsia felis* DNA in the blood of cats and their fleas in the United States. J Feline Med Surg. 2007;9:258–62. DOI: 10.1016/j.jfms.2006.12.005
32. Labruna MB, Ogrzewalska M, Moraes-Filho J, Lep P, Gallegos JL, Lopez J. *Rickettsia felis* in Chile. Emerg Infect Dis. 2007;13:1794–5.
33. Zavala-Velazquez JE, Ruiz-Sosa JA, Sanchez-Elias RA, Becerra-Carmona G, Walker DH. *Rickettsia felis* rickettsiosis in Yucatan. Lancet. 2000;356:1079–80. DOI: 10.1016/S0140-6736(00)02735-5
34. Galvao MA, Zavala-Velazquez JE, Zavala-Castro JE, Mafra CL, Calic SB, Walker DH. *Rickettsia felis* in the Americas. Ann N Y Acad Sci. 2006;1078:156–8. DOI: 10.1196/annals.1374.027
35. Richter J, Fournier PE, Petridou J, Haussinger D, Raoult D. *Rickettsia felis* infection acquired in Europe and documented by polymerase chain reaction. Emerg Infect Dis. 2002;8:207–8.
36. Parola P, Miller RS, McDaniel P, Telford SR III, Rolain JM, Wongsrichanalai C, et al. Emerging rickettsioses of the Thai-Myanmar border. Emerg Infect Dis. 2003;9:592–5.
37. Choi YJ, Jang WJ, Ryu JS, Lee SH, Park KH, Paik HS, et al. Spotted fever group and typhus group rickettsioses in humans, South Korea. Emerg Infect Dis. 2005;11:237–44.
38. Znazen A, Rolain JM, Hammami A, Jemaa MB, Raoult D. *Rickettsia felis* infection, Tunisia. Emerg Infect Dis. 2006;12:138–40.
39. Phongmany S, Rolain JM, Phetsouvanh R, Blacksell SD, Soukkhaseum V, Rasachack B, et al. Rickettsial infections and fever, Vientiane, Laos. Emerg Infect Dis. 2006;12:256–62.

40. Bernabeu-Wittel M, del Toro MD, Nogueras MM, Muniain MA, Cardenosa N, Marquez FJ, et al. Seroepidemiological study of *Rickettsia felis*, *Rickettsia typhi*, *and Rickettsia conorii* infection among the population of southern Spain. Eur J Clin Microbiol Infect Dis. 2006;25:375–81. DOI: 10.1007/s10096-006-0147-6

Address for correspondence: Jorge E. Zavala-Castro, Facultad de Medicina, Unidad Interinstitucional de Investigación Clínica y Epidemiológica, Universidad Autónoma de Yucatán, Avenida Itzaes No. 498 x 59 y 59A Centro, CP 97000, Merida, Yucatán, México; email: zcastro@uady.mx

Household Responses to School Closure Resulting from Outbreak of Influenza B, North Carolina

April J. Johnson,* Zack S. Moore,*† Paul J. Edelson,* Lynda Kinnane,‡ Megan Davies,*† David K. Shay*, Amanda Balish,* Meg McCarron,* Lenee Blanton,* Lyn Finelli,* Francisco Averhoff,* Joseph Bresee,* Jeffrey Engel,† and Anthony Fiore*

School closure is a proposed strategy for reducing influenza transmission during a pandemic. Few studies have assessed how families respond to closures, or whether other interactions during closure could reduce this strategy's effect. Questionnaires were administered to 220 households (438 adults and 355 children) with school-age children in a North Carolina county during an influenza B virus outbreak that resulted in school closure. Closure was considered appropriate by 201 (91%) households. No adults missed work to solely provide childcare, and only 22 (10%) households required special childcare arrangements; 2 households incurred additional costs. Eighty-nine percent of children visited at least 1 public location during the closure despite county recommendations to avoid large gatherings. Although behavior and attitudes might differ during a pandemic, these results suggest short-term closure did not cause substantial hardship for parents. Pandemic planning guidance should address the potential for transmission in public areas during school closure.

Human influenza pandemics have occurred 3 times in the past century and are assumed to be recurring biologic events (*1*). Preparation for the next influenza pandemic has become a major focus of public health activities. Use of vaccines containing antigens matched for a pandemic influenza A strain is the best control measure for reducing illness and death during a pandemic (*1*,*2*). However, specific vaccines will take ≥4–6 months to be produced once a pandemic strain is identified. Additionally, sufficient doses of antiviral drugs might not be available to supply treatment and chemoprophylaxis needs (*3*). As a result, a variety of nonpharmaceutical interventions (NPIs) have been proposed by US federal agencies (*4*) and the World Health Organization (*5*) to help mitigate the impact of a pandemic while vaccines against the pandemic strain are being produced. NPIs that have been identified as potential mitigation strategies include voluntary isolation of case-patients, voluntary quarantine of contacts of cases, and social distancing of children and adults.

School-age children have the highest attack rates during typical seasonal influenza outbreaks and play a central role in sustaining influenza transmission (*6*). Children are being disproportionately affected by the avian influenza A virus (H5N1) that is currently circulating in many countries (*7*). School attendance during the 1957 epidemic was thought to amplify the transmission of virus in Japan (*8*). Proposed mitigation strategies have thus focused on this age group as a means of reducing transmission. School closure lasting 4–12 weeks has been recommended as an option to distance children and decrease transmission (*4*). Several modeling studies have suggested that school closure might reduce peak attack rates and overall clinical attack rates, especially if combined with other strategies, including voluntary isolation and quarantine of sick persons and their contacts (*9*) or household-based antiviral prophylaxis and quarantine (*10*). However, few data are available to address whether school closure can actually reduce the transmission of influenza viruses among susceptible children or their family contacts (*11*).

Prolonged school closures might have adverse social and economic effects (*12*,*13*). For example, some parents

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †North Carolina Department of Health and Human Services, Raleigh, North Carolina, USA; and ‡Toe River Health District, Burnsville, North Carolina, USA

DOI: 10.3201/eid1407.080096

will likely stay home to care for children, resulting in lost family income, as well as adverse effects on businesses. Children from families of lower socioeconomic status may rely on their schools for the National School Lunch Program, a federally assisted meal program that provides meals and snacks to children who qualify (*13*). To date, no study has evaluated parental attitudes or responses to school closures during a seasonal influenza outbreak.

In late October, 2006, a rural county in North Carolina experienced an influenza B virus outbreak that resulted in a sudden increase in student and school staff absenteeism. School officials closed all 9 schools in the county on November 2, and schools remained closed through November 12. These events provided an opportunity to evaluate the response of families with schoolchildren to closing schools and to observe the frequency of children's excursions to public places during the school closure.

Methods

Detection of and Response to the Outbreak

Yancey County, North Carolina, had an estimated population in 2006 of 18,421, of whom ≈21% were <18 years of age, and an estimated 7,472 households in 2000. This county is located in the western part of the state in the Appalachian Highlands on the Tennessee border. From October 26 through November 1, 2006, school officials observed a marked increase in the number of student and employee absences in the 9-school system. Many absences were attributed to influenza-like illness (ILI) among children and staff. Two elementary schools were particularly affected, with absentee rates among students increasing from 4% and 8.8% on October 26 to 34% and 32%, respectively, on November 1. Using commercial rapid antigen detection techniques, a local clinic identified influenza in 29 patients on October 31. The North Carolina Public Health Laboratory subsequently confirmed the presence of influenza B virus in samples that were submitted for viral culture from 7 of 8 children. On November 1, with 429 children (17% of schoolchildren enrolled in all 9 schools; Figure 1, panel A) and 38 (10%) of the staff absent, school officials closed all the county's public schools because of unmet staffing needs.

In an increased effort to vaccinate residents in response to the outbreak, influenza vaccine clinics were established at the county health department. A reverse 911 call was issued to county residents by the health department and county government on November 1. The reverse 911 system is a notification system that enables town officials to deliver telephone messages during an emergency to specific groups of persons on the basis of location. County residents were given the following message by telephone: "This is a message from Yancey County Health Department and Yancey County Government. Due to increasing cases of influenza, residents of Yancey County are being asked to avoid large gatherings. Also, please wash hands frequently, cover coughs, and avoid contact with sick individuals. For more recorded information call ..."

Household Survey

A total of 1,750 households had children enrolled in the public school system. To evaluate the response of Yancey County residents with children in the public school system to the influenza outbreak and school closings, a random sample of these households was contacted by telephone on November 16–18. A standard questionnaire was used. A parent or legal guardian from each household was asked to provide information about his or her child's (or children's) activities during the school closure (November 2–12, 2006), special arrangements that had to be made for child care, and attitudes toward the closure. This household respondent was also asked to provide demographic information on the household and answer questions about how school closure affected his or her own employment and daily routines, and those of any other adults in the household. Respondents were asked their perceptions of the likelihood that a child

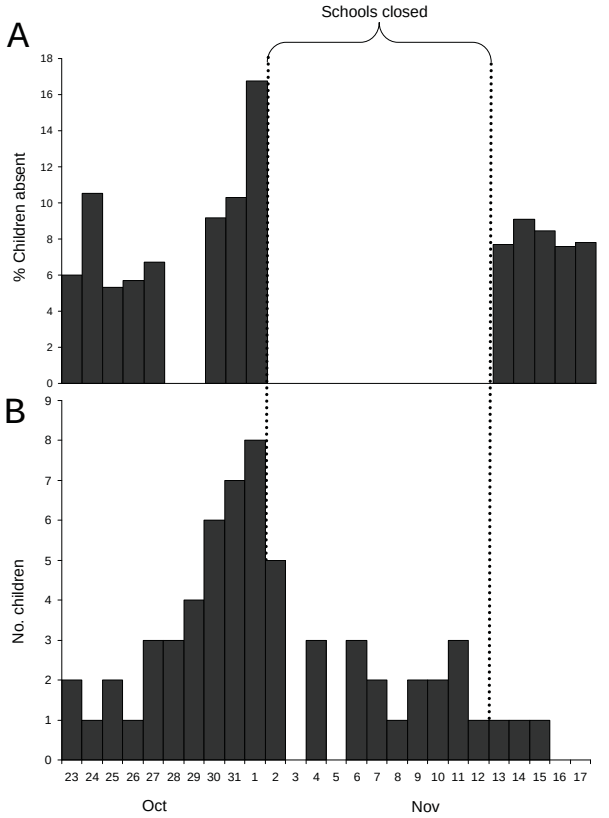


Figure 1. A) Percentage of schoolchildren absent from public schools, by date, and B) total number of children surveyed with influenza-like illness, by date of illness onset, Yancey County, North Carolina, October 23–November 17, 2006.

might be infected with influenza virus and the likelihood that an infected child would require hospitalization. Parents with children who had been ill at some time since October 15, 2006, were asked about clinical manifestations of each child's illness. ILI was defined as having a fever (objective or subjective) and either a cough or sore throat in the absence of a known cause other than influenza.

Four hundred telephone numbers were randomly selected from a list of all households with children enrolled in public schools, with the intent of obtaining ≥200 completed surveys, which would represent >10% of the households and ≥10% of children enrolled in public schools. Families were called at various times of the day and evening both during the week and on the weekend from November 16–18. Information was collected on each school-age child living in the household. To control for the effect of family, 1 child was randomly selected from each household. Statistical analysis was performed by using SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

Interviewers called households over a 3-day period until a minimum of 200 surveys were completed. Two hundred twenty (97%) of those contacted completed the survey; 8 (3%) households refused. The 220 households surveyed included 438 adults (≈3% of the adult population in the county) and 355 school-age children (≈14% of all children enrolled in the public schools, or 9.4% of all children <18 years of age in the county). The percentage of children surveyed from any 1 school ranged from 9% to 21%. The percentage of children in each grade who were surveyed ranged from 8% of third-graders to 20% of ninth-graders. Characteristics of the household and children in the survey are shown in Table 1. Thirty-seven (17%) of the 220 households had only 1 adult (lower than the national rate of single-parent homes, which is 27%). Children from 87 (41%) of 212 responding households were reported as receiving free or reduced-cost lunches through the National School Lunch Program. This finding is slightly lower than the percentage of children reported as enrolled in the county (51%) or the state (48%) in 2005 (*14*), but approximately the same as the national (37.8%) percentage of children eligible for the program (*15*).

One hundred thirty (37%) of 355 surveyed schoolchildren were ill on ≥1 days from October 23 through November 15. Among children who attended elementary schools, 50% were ill during this period, compared with 26% and 28% of surveyed middle and high school-age children, respectively (Table 2). Among children who were reported ill, 66 (51%) met the case definition for ILI. The reported dates for ILI symptom onset started October 20 and peaked on November 1, 2006, the day before schools closed (Figure 1, panel B). A total of 78 (22%) children reportedly received influenza vaccine for that season as of November 15. Sixty-three (81%) of these children received it after schools closed (November 2 or later).

Table 1. Characteristics of children and households surveyed, Yancey County, North Carolina, 2006*

Characteristic	Value
Households (N = 220)	
Single-adult home	37 (17)
Two-adult home	145 (66)
Three- or four-adult home	38 (17)
Children in home receive free/reduced-cost lunch† (n = 212)	87 (41)
All adults employed outside the home	118 (54)
Children (N = 355)	
Male	177 (50)
White, non-Hispanic	344 (97)
Median age, y (range)	12 (5–19)

*Values are no. (%) unless otherwise indicated.
†Free and reduced-cost lunches provided through the National School Lunch Program.

Ninety-nine (45%) household respondents thought it was very possible that their child could get influenza from another person. However, only 15 respondents (7%) felt it was very possible that their child would need hospitalization if they became infected with influenza.

After we controlled for the effect of family, visiting public locations during the school closure (November 2–12) was commonly reported, with 195 (89%) of 220 children visiting at least 1 public place (Table 3). Overall, 47% of children traveled outside Yancey County at least once during the school closure. Sites children visited during school closure differed by age group and illness status. For example, older children were significantly more likely to go to fast food restaurants and parties ($p<0.05$; Figure 2). However, they were less likely to go grocery shopping than younger children. No differences were seen between children who were reported as ill at any time from October 23 through November 15 and children who were not ill during that time (Figure 3).

Among adults in surveyed households, 315 (72%) were employed outside the home (Table 4). In 118 (54%) of the 220 households surveyed, all adults in the household were employed outside the home. Of 218 adults living in those 118 homes, only 39 (18%) had occupations that permitted them to work from home. Seventy-six (24%) of the 315 employed adults missed ≥1 day of work from October 23 through the date of the survey, including 36 (47%) because of their own illness, 18 (24%) to take care of ill

Table 2. Prevalence of child illness reported by parents by school type, Yancey County, North Carolina, 2006

School type	No. (%) surveyed of all public schoolchildren	No. (%) ill of those surveyed
Elementary	136 (12)	68 (50)
Middle	86 (14)	22 (26)
High	128 (16)	36 (28)

Table 3. Locations visited by schoolchildren when schools were closed, controlled for effect of family, Yancey County, North Carolina, 2006

Location visited (N = 220)	No. (%) children
At least 1 public location	195 (89)
Grocery stores	97 (44)
Fast food restaurants	77 (35)
Church services	75 (34)
Mall	42 (19)
Parties or sleepovers	33 (15)
Outside Yancey County	103 (47)

family members, and 14 (18%) because of school closure. However, all adults who reported missing work because of school closure were school employees. Among all adults who missed work during the period, the median number of days missed was 3, and days missed ranged from 1 to 14.

One hundred sixty-seven (76%) households indicated that someone was regularly available during the day to provide childcare (Table 4). Twenty-two (10%) reported that they had to make special childcare arrangements because of school closure, including enlisting working adult household members, grandparents or other relatives, friends, or nonrelated adults to provide childcare; taking the child to work; having older siblings watch younger children; or using childcare programs. Among responding households, only 7 (3%) had to have their child spend ≥1 nights outside their household for childcare purposes, and only 2 (1%) reported having to spend extra money ($100 and $150) on childcare arrangements.

A total of 201 (91%) responding households thought the decision to close schools was appropriate (Table 5). Eighty-two (41%) of households that provided a reason for that opinion thought the decision was appropriate to protect the health of the community, 71 (35%) felt there were too many sick children for schools to remain open, 23 (11%) thought it would help protect their child and family, and 8 (4%) thought that schools would be too understaffed if they remained open. Of the 10 (5%) respondents who believed school closure was inappropriate, 4 (40%) thought it could result in lost income, 3 (30%) did not think influenza was in the area, 2 (20%) did not think closure was an effective measure, and 1 (10%) found it too difficult to make childcare arrangements. Overall, 198 (90%) thought they had enough time to prepare for school closures. A total of 180 (84%) felt well prepared and could not think of anything that could have helped them prepare better to deal with closure. Twenty (9%) would have appreciated more time between notification and closing. Several respondents also mentioned that they would have liked to have been warned that influenza was in the area, that children at school were sick, and that schools might be closed as a response to illnesses.

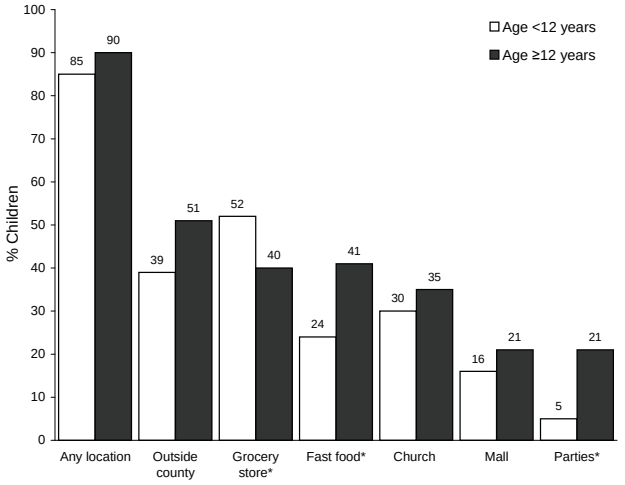


Figure 2. Locations visited by schoolchildren during school closure by age group, controlled for effect of family, Yancey County, North Carolina, 2006. Values above bars are percentages. *p<0.05.

Discussion

The primary objective of this investigation was to evaluate the response of households to school closure caused by an influenza outbreak. This study found that most adults believed that school closure was appropriate and necessary to slow influenza transmission and protect the health of the community. Second, almost all children visited public areas within the community while schools were closed, despite public health recommendations to avoid large gatherings. Lastly, the effect of school closure on work absenteeism and childcare expenditures appeared to be minimal in this community.

Yancey County is located in the Blue Ridge Mountains, and results obtained there are likely not generalizable to all counties. First, residents in this rural, mountainous county are accustomed to dealing with frequent school closures resulting from adverse weather conditions, particularly winter snowstorms. Families in communities where school closures are infrequent or where extended families are less available to provide childcare might respond differently. Additionally, only 17% of households in this survey were single-adult homes, compared with the national average of 27%. Multiple-adult households might find arranging childcare for schoolchildren during school closures to be less challenging than single-adult homes. Also, the median age of children in this survey was 12 years. Childcare arrangements for older children are likely easier to make than for younger children. Lastly, no adults in this survey reported missing work solely because of school closure, other than those employed by the school. Only 18 (8%) adults from the 220 households in the survey reported missing work to stay with a sick family member. This finding is

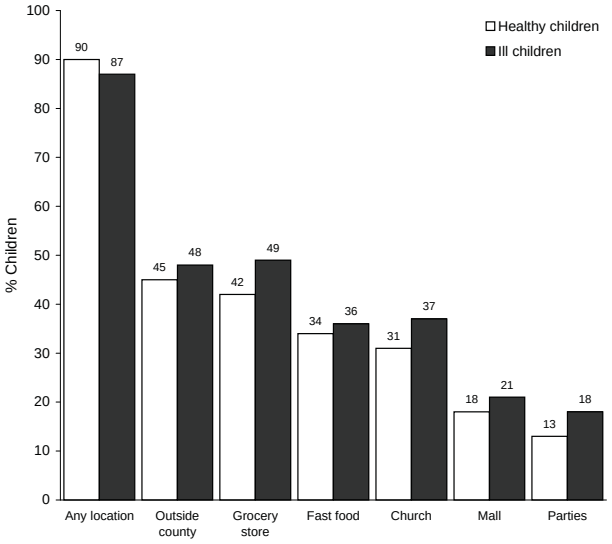


Figure 3. Percentage of ill and healthy schoolchildren visiting various locations during school closure, controlled for effect of family, Yancey County, North Carolina, 2006. Values above bars are percentages. No significant differences were observed (p<0.05).

dissimilar to findings from another study that found that at least 1 adult in 53% of families missed work to care for an ill child because of a winter respiratory illness (*16*). Other studies have also found that epidemics of respiratory illness can cause a substantial number of lost workdays for parents of ill children (*17*,*18*).

Results might also have been different if schools were closed for a longer period or if a more clinically severe strain of influenza were present, causing more hospitalizations or death. Most parents interviewed in the present study did not think that infection would result in hospitalization; only 5 brief hospitalizations were reported. We did not collect quantitative information on the frequency or duration of visits to public places by schoolchildren and did not determine whether these persons visited public places while potentially infectious. Lastly, households with children who attended private school or were home-schooled were not surveyed.

The decision of the local school board to close all 9 schools in Yancey County was primarily motivated by concerns about staffing the schools in the face of high levels of absenteeism. Although the reduction in ILI that occurred after schools were closed is an intriguing finding, results from this investigation cannot be used to assess the effect of school closure on the effect of illness in a community experiencing an influenza outbreak. Influenza outbreak dynamics are relatively poorly understood, and the proportion of children who were susceptible to infection might have decreased below the number required to sustain transmission at approximately the same time schools were closed. The fact that transmission decreased despite many schoolchildren in public gathering places also calls into question the role of reduced contacts among children in ending the outbreak.

Studies that have modeled the effects of NPIs on reducing influenza epidemic size during a pandemic have suggested that closing schools can be effective if implemented early and if the reproductive number (R_0) is low (<1.8) (*19*). For example, in a network-based simulation in which ≈50% of persons were infected, similar to the Asian influenza pandemic of 1957–58, closing schools and keeping those children at home reduced the calculated attack rate by 90% (*20*). Studies of the effects of school closure on respiratory disease rates in schoolchildren have shown mixed results. An investigation in Israel showed that al-

Table 4. Employment and childcare status of adults and households during school closure, Yancey County, North Carolina, 2006*

Characteristic	Value
Adults employed outside home (N = 438)	315 (72)
Occupations of those employed outside home (N = 315)	
Healthcare	35 (11)
Education	36 (11)
Industry	27 (9)
No. homes where all adults in home employed (N = 220)	118 (54)
Adults who can work from home (in homes where all adults in the house work outside the home, N = 218)	39 (18)
Missed worked from Oct 23 through Nov 15 (N = 315)	76 (24)
Median no. days missed (range)	3 (1–14)
Reason for missed work (N = 76)	
Own illness	36 (47)
Had to take care of ill family members	18 (24)
School closure	14 (18)
Childcare (N = 220)	
Someone home during the day who could provide childcare	167 (76)
Had to make special arrangements	22 (10)
Child had to spend ≥1 nights away from home for childcare purposes	7 (3)
Had to spend extra money for childcare	2 (1)

*Values are no. (%) unless otherwise indicated.

Table 5. Household responses to school closure and difficulties obtaining childcare, Yancey County, North Carolina, 2006

Response	No. (%)
Felt closure was appropriate (N = 220)	201 (91)
Reason it was appropriate (N = 201)	
To protect health of the community	82 (41)
Too many sick children	71 (35)
To protect their child and family	23 (11)
Schools would be understaffed	8 (4)
Felt closure was not appropriate (N = 220)	10 (5)
Reason it was not appropriate (N = 10)	
Could result in lost income	4 (40)
Did not think influenza was in the area	3 (30)
Did not think it was an effective measure	2 (20)
Too difficult to make childcare arrangements	1 (10)
Overall preparedness (N = 220)	
Had enough time to prepare for closure	198 (90)
Could not think of anything that would have made them more prepared	180 (84)
Could have used more time	20 (9)

though schools were closed because of a teacher strike, the incidence of respiratory illness diagnosed in children who came to health clinics decreased, as did physician and emergency department visits and purchase of medications (*11*). Rates subsequently increased when schools reopened. However, some (researchers and public health officials) have proposed that school closure in urban areas might have an opposite effect because children released from school can more easily congregate. This effect may have occurred in children from Chicago during the 1918–19 pandemic when rates of influenza among schoolchildren increased during time off from school (*21*). In Connecticut, 3 cities (Bridgeport, Hartford, and New Haven) kept their schools open during the 1918–19 pandemic (*21*) and experienced lower mortality rates than 2 smaller cities (New London and Waterbury) that closed schools.

We cannot assess the effect of influenza vaccination on the course of the influenza outbreak in Yancey County. Large-scale vaccination programs began in the county during late October. Although not known at the time of these programs, the influenza B strain contained in the 2006–2007 vaccine (Victoria lineage) was not a good match to the circulating Yamagata lineage influenza B viruses in this outbreak (*22*). However, only a few influenza virus isolates were antigenically characterized at the Centers for Disease Control and Prevention (CDC) in Atlanta, and we do not know that the isolates tested were representative of those circulating in the area at the time. Influenza A viruses were found through CDC's sentinel surveillance systems to be circulating in other counties concurrently. It is possible that other viruses were co-circulating in Yancey County and were not detected.

The effectiveness of closing schools to reduce transmission of common infectious diseases such as influenza is not well studied (*12*). In addition, data on school closures in response to infectious disease outbreaks in general are not regularly collected in the United States. Additional studies to assess these actions will be of interest to local public health officials and school administrators who make decisions about keeping schools open during explosive but self-limited outbreaks with high attack rates among schoolchildren, such as those commonly caused by seasonal influenza or norovirus. Results from such studies will also be helpful in planning and implementing community mitigation strategies for disease outbreaks whose community impact might be severe, such as pandemic influenza.

This investigation provides insight into how households with school-age children in a small rural community responded to a brief school closure precipitated by a seasonal influenza outbreak. Overall, respondents to the survey indicated that the community was in favor of closing schools as a way to deal with high levels of student and staff absenteeism and potentially to reduce transmission. Parents reported few problems in coping with the school closure and did not miss work to provide childcare. However, many students visited public areas during school closure. Plans for pandemic influenza responses should address the potential for transmission in public areas during school closure.

Acknowledgments

We thank Mary Alice Beaver and Darlene Tipton for assistance during the investigation; the North Carolina State Laboratory of Public Health for laboratory support; and Kirsten Buckley, Christina Felsen, Amanda McWhorter, John Turnbull, Daiscia Roseberry, Emmanuel Johnson, Vivek Shinde, Nathan Almond, Meredith Anderson, David Bergmire-Sweat, Kim Brunette, Andrew Edmonds, Anjum Hajat, Jen Horney, Morgan Johnson, Tina Lusk, Molly McKnight, Meagan Pressley, Mindi Russell, Lindsay Tallon, and John Wallace for assistance with data collection and entry.

Dr Johnson is an Epidemic Intelligence Service officer in the Influenza Division at the Centers for Disease Control and Prevention. Her research interests are the epidemiology of viral respiratory infections and zoonotic diseases.

References

1. Stöhr K, Esveld M. Will vaccines be available for the next influenza pandemic? Science. 2004;306:2195–6. DOI: 10.1126/science.1108165
2. Sellwood C, Asgari-Jirhandeh N, Salimee S. Bird flu: if or when? Planning for the next pandemic. Postgrad Med J. 2007;83:445–50. DOI: 10.1136/pgmj.2007.059253
3. Germann TC, Kadau K, Longini IM Jr, Macken CA. Mitigation strategies for pandemic influenza in the United States. Proc Natl Acad Sci U S A. 2006;103:5935–40. DOI: 10.1073/pnas.0601266103

4. Centers for Disease Control and Prevention. Community strategy for pandemic influenza mitigation, 2008 [cited 2008 Apr 24]. Available from http://pandemicflu.gov/plan/community/commitigation.html#XV
5. World Health Organization. WHO interim protocol: rapid operations to contain the initial emergence of pandemic influenza, 2007 [cited 2008 Apr 24]. Available from http://www.who.int/csr/disease/avian_influenza/RapidContProtMay07.pdf
6. Monto AS, Davenport FM, Napier JA, Francis T Jr. Effect of vaccination of a school-age population upon the course of an A2/Hong Kong influenza epidemic. Bull World Health Organ. 1969;41:537–41.
7. Chen JM, Chen JW, Dai JJ, Sun YX. A survey of human cases of H5N1 avian influenza reported by the WHO before June 2006 for infection control. Am J Infect Control. 2007;35:467–9. DOI: 10.1016/j.ajic.2006.12.001
8. Reichert TA, Sugaya N, Fedson DS, Glezen P, Simonsen L, Tashiro M. The Japanese experience with vaccinating schoolchildren against influenza. N Engl J Med. 2001;344:889–96. DOI: 10.1056/NEJM200103223441204
9. Haber MJ, Shay DK, Davis XM, Patel R, Jin X, Weintraub E, et al. Effectiveness of interventions to reduce contact rates during a simulated influenza pandemic. Emerg Infect Dis. 2007;13:581–9.
10. Ferguson NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS. Strategies for mitigating an influenza pandemic. Nature. 2006;442:448–52. DOI: 10.1038/nature04795
11. Heymann A, Chodick G, Reichman B, Kokia E, Laufer J. Influence of school closure on the incidence of viral respiratory diseases among children and on health care utilization. Pediatr Infect Dis J. 2004;23:675–7. DOI: 10.1097/01.inf.0000128778.54105.06
12. Inglesby TV, Nuzzo JB, O'Toole T, Henderson DA. Disease mitigation measures in the control of pandemic influenza. Biosecur Bioterror. 2006;4:366–75. DOI: 10.1089/bsp.2006.4.366
13. Pandemic influenza: warning, children at-risk. October 2007 report. Washington: American Academy of Pediatrics and Trust for America's Health; 2007. p. 1–31.
14. The Annie E. Casey Foundation. CLIKS: community-level information on kids, 2006 [cited 2008 Apr 24]. Available from http://www.kidscount.org/cgi-bin/cliks.cgi?action=profile_results&subset=NC&areaid=101
15. Garofono A, Sable J. Characteristics of the 100 largest public elementary and secondary school districts in the United States: 2004–2005 (NCES 2008–335). Washington: National Center for Education Statistics, Institute of Education Sciences, US Department of Education; 2008.
16. Nettleman MD, White T, Lavoie S, Chafin C. School absenteeism, parental work loss, and acceptance of childhood influenza vaccination. Am J Med Sci. 2001;321:178–80. DOI: 10.1097/00000441-200103000-00004
17. Li S, Leader S. Economic burden and absenteeism from influenza-like illness in healthy households with children (5–17 years) in the US. Respir Med. 2007;101:1244–50. DOI: 10.1016/j.rmed.2006.10.022
18. Neuzil KM, Hohlbein C, Zhu Y. Illness among schoolchildren during influenza season; effect on school absenteeism, parental absenteeism from work and secondary illness in families. Arch Pediatr Adolesc Med. 2002;156:986–91.
19. Vynnycky E, Edmunds WJ. Analyses of the 1957 (Asian) influenza pandemic in the United Kingdom and the impact of school closures. Epidemiol Infect. 2008;136:166–79. DOI: 10.1017/S0950268807008369
20. Glass RJ, Glass LM, Beyeler WE, Min HJ. Targeted social distancing design for pandemic influenza. Emerg Infect Dis. 2006;12:1671–81.
21. Bell DM; World Health Organization Writing Group. Non-pharmaceutical interventions for pandemic influenza, national and community measures. Emerg Infect Dis. 2006;12:88–94.
22. Centers for Disease Control and Prevention (CDC). Update: influenza activity—United States and worldwide, 2006–07 season, and composition of the 2007–08 influenza vaccine. MMWR Morb Mortal Wkly Rep. 2007;56:789–94.

Address for correspondence: April J. Johnson, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop A32, Atlanta, GA 30333, USA; email: ajjohnson@cdc.gov

Attributable Outcomes of Endemic *Clostridium difficile*–associated Disease in Nonsurgical Patients

Erik R. Dubberke,* Anne M. Butler,* Kimberly A. Reske,* Denis Agniel,* Margaret A. Olsen,* Gina D'Angelo,* L. Clifford McDonald,† and Victoria J. Fraser*

Data are limited on the attributable outcomes of *Clostridium difficile*–associated disease (CDAD), particularly in CDAD-endemic settings. We conducted a retrospective cohort study of nonsurgical inpatients admitted for ≥48 hours in 2003 (N = 18,050). The adjusted hazard ratios for readmission (hazard ratio 2.19, 95% confidence interval [CI] 1.87–2.55) and deaths within 180 days (hazard ratio 1.23, 95% CI 1.03–1.46) were significantly different among CDAD case-patients and noncase patients. In a propensity score matched-pairs analysis that used a nested subset of the cohort (N = 706), attributable length of stay attributable to CDAD was 2.8 days, attributable readmission at 180 days was 19.3%, and attributable death at 180 days was 5.7%. CDAD patients were significantly more likely than controls to be discharged to a long-term-care facility or outside hospital. Even in a nonoutbreak setting, CDAD had a statistically significant negative impact on patient illness and death, and the impact of CDAD persisted beyond hospital discharge.

Clostridium difficile–associated disease (CDAD) is an increasingly common cause of hospital-associated diarrhea (*1*,*2*). The emerging NAP1 strain of *C. difficile* has been associated with numerous outbreaks and appears to be more virulent than other endemic and epidemic *C. difficile* strains (*3*–*9*). Despite the increasing importance of this pathogen, few data exist on outcomes attributable to CDAD (*10*–*14*). The attributable mortality for CDAD has recently been estimated at 6.9% and 16.7% (*9*,*12*). However, these studies were performed during CDAD outbreaks caused by the NAP1 strain. Published estimates of CDAD-attributable deaths in disease-endemic settings are much lower (1.2%–1.5%) (*10*,*13*). Kyne et al. did not find endemic CDAD to be an independent predictor of death within 1 year of CDAD, but that study was relatively small (47 CDAD cases) (*11*). Thus, additional data with larger sample sizes are needed to determine outcomes associated with CDAD in nonoutbreak settings. With a large cohort of CDAD patients at a tertiary-care center, we evaluated CDAD outcomes including length of stay, hospital discharge status, time-to-readmission, and deaths in a CDAD-endemic setting.

Methods

This study was conducted at Barnes-Jewish Hospital (BJH), a 1,250-bed, tertiary-care academic hospital in St. Louis, Missouri. Eligibility was limited to nonsurgical patients admitted for ≥48 hours from January 1 through December 31, 2003. Nonsurgical patients were defined as those without operating room costs. Surgical patients were excluded because of their heterogeneity. Specifically, risk factors for length of stay, readmission to the hospital, and death were different in this population compared with other hospitalized patients. Data were primarily collected from the hospital's Medical Informatics database. The database was queried to collect patient demographics; admission and discharge dates; International Classification of Diseases, 9th edition, Clinical Modification (ICD-9-CM), diagnosis and procedure codes (online Appendix, available from www.cdc.gov/EID/content/14/7/1031-app.htm); inpatient medication orders; vital signs; and laboratory results, including *C. difficile* toxin assay results. The Medical Informatics database was also queried to ascertain date of death. Patients without a death date in the database were screened for death by reviewing the Social Security Death Index.

*Washington University School of Medicine, St. Louis, Missouri, USA; and †Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: 10.3201/eid1407.070867

For each patient, a modified APACHE II Acute Physiology Score (APS) was calculated to adjust for severity of illness (*15*). The APS was based on laboratory results and vital signs collected within 24 hours of admission. The score was modified because data for respiratory rates and Glasgow coma scores were unavailable electronically. In addition, the Charlson-Deyo method was used to classify co-existing conditions (*16*,*17*). Albumin levels within 24 hours of admission were collected and categorized into normal (>3.5 g/dL), low (2.5–3.5 g/dL), and very low (<2.5 g/dL). Multiple imputation methods were used to impute albumin levels for patients without recorded values (*18*). For CDAD case-patients, only medication and intensive-care unit exposures before the patient's first positive stool toxin assay were included in analyses.

Case Definition

CDAD case-patients were defined as inpatients with positive *C. difficile* stool toxin assays (TechLab, Blacksburg, VA, USA). The microbiology laboratory only performs toxin tests on unformed stool, so all patients with a positive result for toxin were considered case-patients. Both community-onset and hospital-onset CDAD case-patients were included in the analyses.

Analyses were performed on the full cohort and a nested case–control population. The first component was a retrospective cohort. For CDAD patients, the admission date when the patient's CDAD was first identified was used as the index admission. For noncases with >1 admission during the study period, 1 admission was randomly selected as the index admission. The nested case–control population consisted of propensity score matched cases and controls from patients identified in the cohort.

Cohort

Data Analysis

Survival was defined as the number of days from the index hospital admission until death. Survival was censored at 180 days. Time to readmission was calculated as the number of days between the index hospitalization discharge date and the date of the subsequent admission to BJH, if applicable. Days until readmission were censored at death or 180 days, whichever occurred first.

Fisher exact, χ^2, and Mann-Whitney U tests were used to compare characteristics of patients with and without CDAD. Time-to-event methods were used to estimate the effect of CDAD on 180-day survival and time-to-readmission. Patients who died during the index hospitalization were excluded from the time-to-readmission analysis. Kaplan-Meier analysis was used to evaluate the unadjusted relationships between CDAD and time-to-event outcomes. Cox proportional hazards regression was used to estimate unadjusted and adjusted hazard ratios and 95% confidence intervals (CIs). All variables with biologic plausibility or $p \leq 0.15$ in the univariate analysis were considered in the multivariable Cox regression analysis by using backward stepwise selection. Variables were sequentially removed from the final model, starting with the variable most weakly associated with the outcome. The significance of individual covariates was determined by using a Wald statistic of $p \leq 0.05$. The proportional hazards assumption was verified by assessing the parallel nature of curves in log-log plots.

Propensity Score Matched-Pairs Analysis

The second component of this study was a propensity score matched-pairs analysis of outcomes attributable to CDAD. This study design complemented the cohort by enabling analyses that could not be conducted in the entire cohort, specifically hospital discharge status, attributable length of stay, attributable time-to-readmission, and attributable death. Hospital discharge status could not be analyzed for the entire cohort because manual review of medical records was required to determine the discharge location of each patient. The large size of the cohort prohibited this analysis. In addition, survival and time-to-readmission estimates generated in the cohort analysis were validated in the matched-pairs analysis.

Cases and a subset of controls were selected from the primary cohort for the matched-pairs analysis. CDAD case-patients were matched to controls based on their propensity for CDAD to develop. Patient-specific probabilities of developing CDAD were predicted by a full logistic regression model adjusted for all variables suspected to impact the risk of developing CDAD (online Appendix). Variables with $p \leq 0.05$ in univariate analysis or biologic plausibility were included in the full logistic regression model. CDAD case-patients and controls were matched by a 1:1 ratio that used the nearest-neighbor method within calipers of 0.015 standard deviations (*19*). CDAD cases without an available nearest-neighbor control were excluded from the analysis. Chi-square, Fisher exact, and Mann Whitney U tests were used, as appropriate, to compare characteristics of CDAD case-patients and controls.

Medical records were reviewed for all CDAD case-patients and controls to determine hospital discharge location for each patient. Patients were categorized as being discharged to home, to a long-term-care facility, or to an outside hospital or dying in the hospital. Long-term-care facility was defined as a long-term-care facility, long-term acute-care facility/chronic ventilation facility, inpatient rehabilitation facility, skilled nursing facility, or nursing home. Outside hospital was defined as a non-BJH hospital or acute-care facility.

Data Analysis

Median length of stay was determined for CDAD case-patients and controls. The difference in median pairwise length of stay was compared with the Wilcoxon signed-rank test. Attributable length of stay was calculated as the median pairwise difference between CDAD case-patients and controls. Frequencies, adjusted odds ratios, and 95% CIs were calculated to determine if discharge location was associated with CDAD. CDAD-attributable 180-day readmission was calculated as the difference in readmission between CDAD case-patients and controls. Attributable deaths from 0–180 days, 0–60 days, and 61–180 days after admission were also calculated by using this method.

The primary survival endpoints of interest were death and readmission, which were both censored at 180 days or at death. Kaplan-Meier analyses, conducted by using log-rank tests, were used to determine relationships between the survival endpoints and CDAD. Cox proportional hazards regression stratified by matched-pairs was used to obtain hazard ratios and 95% CIs. Violation of the proportional hazards assumption was verified by the crossing nature of curves in the log-log plots. Therefore, we used an extended Cox regression model stratified by matched-pairs for the periods ≤60 days and >60 days. The breakpoint of 60 days was chosen because the graph of survival curves for CDAD case-patients and controls diverged at ≈60 days. Violation of the proportional hazards assumption was confirmed by the significance of the coefficient for the product term between CDAD and ≤60 days and >60 days (*20*).

All tests were 2-tailed, and $p \leq 0.05$ was considered significant. Statistical analyses were performed with SPSS for Windows version 14.0 (SPSS, Inc., Chicago, IL, USA) and SAS version 9.1 (SAS Institute, Cary, NC, USA). The Washington University Human Studies Committee approved this project.

Results

Among 18,050 nonsurgical inpatients admitted during the 1-year study period, 390 had CDAD and 17,660 did not. Selected patient characteristics of the cohort are summarized in Table 1. CDAD patients were significantly older (median 66.0 vs. 52.7 years, $p<0.001$) more likely to be men, and more likely to be Caucasian than were non-case-patients. CDAD case-patients had a higher severity of illness on admission than noncases, as indicated by the modified APS. CDAD patients were more likely to have a diagnosis of congestive heart failure, chronic obstructive

Table 1. Baseline characteristics of study cohort, *Clostridium difficile*–associated disease (N = 18,050)*

Characteristic	CDAD case-patients (n = 390), no. (%)	Non–case-patients (n = 17,663), no. (%)	p value†
Age, y			
<45	58 (15)	6,847 (39)	<0.001
45–65	132 (34)	5,187 (29)	0.06
>65	200 (51)	5,626 (32)	<0.001
Male sex	194 (50)	6,704 (38)	<0.001
White race	257 (66)	9,860 (56)	<0.001
Modified APS			
≤2	77 (20)	6,687 (38)	<0.001
3–4	76 (20)	4,573 (26)	0.004
5–6	82 (21)	2,970 (17)	0.028
≥7	155 (40)	3,430 (19)	<0.001
Liver disease			
Mild	5 (1)	204 (1)	0.77
Moderate to severe	6 (2)	209 (1)	0.47
Diabetes without chronic complications	70 (18)	2,718 (15)	0.17
Diabetes with chronic complications	15 (4)	416 (2)	0.06
Myocardial infarction	26 (7)	1466 (8)	0.25
Congestive heart failure	97 (25)	2,562 (15)	<0.001
Cerebral vascular disease	16 (4)	882 (5)	0.42
Chronic obstructive pulmonary disease	90 (23)	2,564 (15)	<0.001
Rheumatologic/collagen vascular disease	11 (3)	361 (2)	0.29
Peptic ulcer disease	5 (1)	279 (2)	0.64
Cancer, excluding leukemia or lymphoma	45 (12)	1,283 (7)	0.001
Leukemia or lymphoma	69 (18)	567 (3)	<0.001
Metastatic solid tumor	33 (9)	936 (5)	0.01
HIV/AIDS	5 (1)	209 (1)	0.81
Paraplegia or hemiplegia	8 (2)	223 (1)	0.17

*CDAD, *Clostridium difficile*–associated disease; APS, Acute Physiology Score.
† Fisher exact test, χ^2 test.

pulmonary disease, cancer, leukemia or lymphoma, and metastatic solid tumors.

Of 17,492 patients alive at the index hospitalization discharge, 4,207 (24%) were readmitted to BJH within 180 days. Fifty-two percent of CDAD patients were readmitted within 180 days versus 23% of noncases (log-rank p<0.001). Univariate and multivariable Cox regression results for time to readmission are presented in Table 2. The adjusted hazard ratio (HR) for readmission within 180 days was significantly higher for CDAD case-patients than noncases (HR 2.19, 95% CI 1.87–2.55) (Table 2).

By 180 days after hospital admission, 149 (38%) of 390 CDAD case-patients and 2,150 (12%) 17,660 noncase-patients had died. In the Kaplan-Meier analysis, the mortality rate was significantly higher for CDAD case-patients than noncases (log rank p<0.001) (Figure 1). Unadjusted and adjusted Cox regression results for death within 180 days of admission ("180-day mortality") are presented in Table 3. The adjusted hazard ratio for 180-day mortality was significantly higher for CDAD case-patients than noncase patients (HR 1.23, 95% CI 1.03–1.46) (Table 3).

The propensity score matched-pairs analysis included 353 CDAD cases and 353 controls (N = 706). There were no significant differences between the matched cases and controls after correcting for multiple testing with the Bonferroni procedure. Thirty-seven CDAD case-patients were

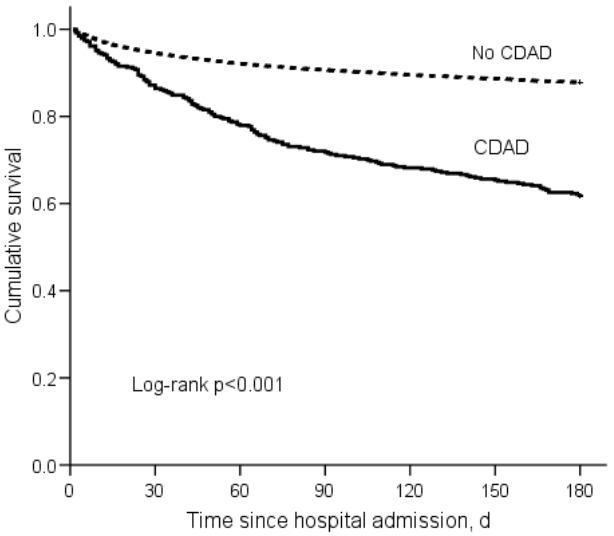


Figure 1. Kaplan-Meier survival estimates for cohort (N = 18,050). CDAD, *Clostridium difficile*–associated disease.

Table 2. Cox proportional hazards estimate of readmission at 180 d in *Clostridium difficile*–associated disease (CDAD) study cohort (N = 17,492; 4,207 readmissions, 13,285 censored)*†

Variable	Univariate hazard ratio‡ (95% CI)	Multivariable hazard ratio ‡ (95% CI)
CDAD	3.09 (2.95–3.23)	2.19 (1.87–2.55)
Male sex	1.42 (1.40–1.45)	1.11 (1.05–1.19)
White race	1.26 (1.23–1.28)	1.06 (1.00–1.13)
Modified APS		
≤2	Reference	Reference
3–4	1.15 (1.12–1.18)	1.10 (1.02–1.20)
5–6	1.39 (1.35–1.43)	1.24 (1.13–1.35)
≥7	1.84 (1.80–1.89)	1.50 (1.37–1.64)
Albumin, g/dL§		
>3.5	Reference	Reference
2.5–3.5	1.05 (1.03–1.08)	0.99 (0.92–1.08)
<2.5	1.03 (0.99–1.07)	0.95 (0.80–1.14)
Liver disease		
None	Reference	Reference
Mild	1.80 (1.67–1.94)	1.44 (1.12–1.83)
Moderate to severe	1.79 (1.65–1.94)	1.48 (1.13–1.93)
Diabetes with chronic complications	1.89 (1.80–1.99)	1.53 (1.30–1.80)
Diabetes without chronic complications	1.29 (1.26–1.32)	1.10 (1.02–1.19)
Congestive heart failure	1.60 (1.56–1.64)	1.34 (1.23–1.45)
Cerebrovascular disease	0.77 (0.74–0.81)	0.74 (0.63–0.87)
Cancer, excluding leukemia or lymphoma	2.75 (2.67–2.83)	1.90 (1.70–2.13)
Leukemia or lymphoma	2.31 (2.18–2.45)	1.84 (1.52–2.23)
Metastatic solid tumor	2.81 (2.71–2.91)	1.66 (1.46–1.90)
HIV/AIDS	1.74 (1.62–1.87)	1.74 (1.38–2.19)
ICU admission	1.06 (1.03–1.09)	0.84 (0.76–0.93)

*CI, confidence interval; APS, Acute Physiology Score; ICU, intensive care unit.
†The analysis excluded 558 patients who died during the index hospital admission. Nonsignificant variables considered in the model included mechanical ventilation, paraplegia/hemiplegia, chronic obstructive pulmonary disease, myocardial infarction, rheumatologic/collagen vascular disease, and peptic ulcer disease.
‡Hazard ratios also adjusted for categorical age (<20, 20–24, 25–29, 30–34, 35–39, 40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, 75–79, 80–84, 85–89, 90–94, ≥95 y).
§7,610 (42%) patients were missing albumin values. Values were imputed by using multiple imputation methods.

Table 3. Cox proportional hazards estimate of deaths from *Clostridium difficile*–associated disease (CDAD) at 180 d in study cohort (N = 18,050; 2,299 deaths, 15,751 censored)*†

Variable	Univariate hazard ratio‡ (95% CI)	Multivariable hazard ratio‡ (95% CI)
CDAD	3.55 (3.37–3.75)	1.23 (1.03–1.46)
Male sex	1.73 (1.68–1.77)	1.17 (1.08–1.27)
White race	1.65 (1.61–1.70)	1.22 (1.11–1.33)
Modified APS		
≤2	Reference	Reference
3–4	1.41 (1.36–1.47)	1.09 (0.96–1.24)
5–6	2.09 (2.00–2.17)	1.30 (1.14–1.49)
≥7	4.11 (3.97–4.25)	1.65 (1.46–1.87)
Albumin, g/dL§		
>3.5	Reference	Reference
2.5–3.5	2.12 (1.90–2.36)	1.62 (1.45–1.82)
<2.5	4.77 (3.91–5.81)	2.93 (2.52–3.42)
Liver disease		
None	Reference	Reference
Mild	3.08 (2.86–3.33)	2.37 (1.85–3.04)
Moderate to severe	5.50 (5.17–5.85)	3.76 (3.05–4.64)
Diabetes with chronic complications	1.47 (1.37–1.58)	1.49 (1.18–1.88)
Congestive heart failure	1.85 (1.80–1.91)	1.28 (1.15–1.42)
Cerebrovascular disease	1.68 (1.60–1.76)	1.62 (1.37–1.92)
Cancer, excluding leukemia or lymphoma	6.42 (6.24–6.61)	2.44 (2.15–2.76)
Leukemia or lymphoma	3.17 (2.99–3.38)	4.92 (3.98–6.08)
Metastatic solid tumor	8.82 (8.57–9.09)	4.41 (3.87–5.03)
HIV/AIDS	1.77 (1.62–1.95)	2.88 (2.12–3.91)
Paraplegia/ hemiplegia	1.75 (1.60–1.92)	1.53 (1.12–2.07)
Mechanical ventilation	6.39 (6.18–6.62)	3.17 (2.71–3.71)
ICU admission	3.08 (2.99–3.17)	1.31 (1.14–1.50)

*CI, confidence interval; APS, Acute Physiology Score; ICU, intensive care unit.
†Nonsignificant variables considered in the model included diabetes without chronic complications, chronic obstructive pulmonary disease, myocardial infarction, rheumatologic/collagen vascular disease and peptic ulcer disease. Of 2,299 people who died within 180 d of admission, 1,565 (68%) deaths were identified by means of the hospital Medical Informatics database and 734 (32%) were identified with the Social Security Death Index.
‡Hazard ratios also adjusted for categorical age (<20, 20–24, 25–29, 30–34, 35–39, 40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, 75–79, 80–84, 85–89, 90–94, ≥95 y).
§7,525 (43%) patients were missing albumin values. Values were imputed by using multiple imputation methods.

dropped because a nearest-neighbor control was not available. Unmatched CDAD patients had significantly higher modified APS (median = 7.0 vs. 5.0, p<0.001), longer median length of stay (13.6 days vs. 9.6 days, p = 0.01), and higher percentage of deaths at 180 days (59% vs. 36%, p = 0.01) than matched case-patients.

In the matched-pairs analysis, median length of stay was 9.6 days for CDAD patients compared with 5.8 days for controls, and the increased attributable length of stay for CDAD patients was 2.8 days (Wilcoxon signed-rank p<0.001). Among the 706 patients in the matched-pairs analysis, 445 (63%) were discharged to home and 188 (27%) were discharged to a long-term-care facility. Only 7 (1%) patients were discharged to an outside hospital; therefore, these patients were combined with patients discharged to a long-term-care facility in the analysis. CDAD patients were significantly more likely than controls to be discharged to a long-term-care facility or outside hospital (32% vs. 23%, odds ratio 1.62, 95% CI 1.15–2.28, McNemar p = 0.01).

Among 290 matched-pairs with both patient and control alive at index hospitalization discharge, 148 CDAD patients were readmitted to BJH within 180 days compared with 92 controls, for an attributable readmission of 19.3% (11.4%–27.2%). In the Kaplan-Meier and Cox model analyses, CDAD patients were significantly more likely than controls to be readmitted to the hospital within 180 days (Figure 2, Table 4).

By 180 days after hospital admission, 127 CDAD patients died compared with 107 controls, for an attributable mortality of 5.7% (95% CI –1.3%–12.6%). Although CDAD case-patients were no more likely than controls to die within 60 days of hospital admission, a divergence in survival between CDAD case-patients and controls began 60 days after hospital admission (Figure 3, Table 4). The HR for death from 61–180 days was significantly higher for CDAD patients than controls (HR 2.00, 95% CI 1.47–2.72) (Table 4). Among 223 matched-pairs with both case-patients and controls alive after day 60, 19.7% of CDAD patients and 12.6% of controls died within 180 days for an attributable mortality between 61–180 days of 7.2% (95% CI 0.4%–14.0%).

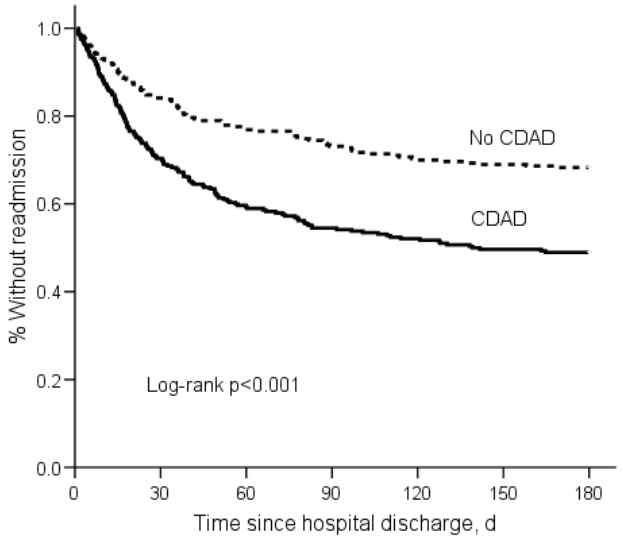


Figure 2. Kaplan-Meier estimates of time until hospital readmission for matched pairs (n = 580). CDAD, *Clostridium difficile*–associated disease.

Discussion

The results of this study indicate that CDAD is a major contributor to death even in nonoutbreak settings. In this CDAD-endemic setting, the disease was associated with a 23% higher hazard of death within 180 days after hospital admission in the multivariable cohort analysis and a 7.2% attributable mortality 61–180 days after hospital admission in the matched-pairs analysis. Historically, endemic CDAD has been reported to be associated with minimal increased risk in mortality although NAP1 strain CDAD outbreaks have been associated with much higher attributable mortality (*10*,*11*,*13*). Two studies of CDAD in endemic settings reported 1.2%–1.5% inhospital mortality rates from CDAD (*10*,*13*). Using a multivariable Cox proportional hazards model, Kyne et al. found no association between CDAD and 1-year mortality, although that study was quite small (47 CDAD patients) (*11*). In contrast, several studies have identified increased deaths associated with outbreaks of the NAP1 strain. Pepin et al. estimated the 1-year attributable mortality of CDAD during an outbreak with the NAP1 strain to be 16.7% (*9*). Hubert et al. reported that CDAD was the attributable or contributive cause of death in 8.4% of patients infected with a strain of *C. difficile* that had the binary toxin and *tcdC* deletion (*21*). Loo et al. found CDAD to be the attributable cause of death within 30 days in 6.9% of CDAD patients and suspected that CDAD contributed to death in another 7.5% of CDAD patients (*12*). The estimate of 6.9% attributable mortality, however, was determined through chart review, not through multivariable analyses, and medical chart review may not be an adequate method to determine attributable mortality because of subjectivity (*22*).

Although the 5.7% 180-day attributable mortality determined in the propensity score matched-pairs analysis in our study was not statistically significant, the estimate is substantially higher than estimates reported from other CDAD-endemic settings. The attributable mortality we report is more consistent with estimates from outbreaks of the NAP1 strain and is likely clinically significant. The NAP1 strain was first identified at BJH during 2005, but the strain may have been present during the study period (*23*). During the years 2000–2006 at BJH, there were no apparent increases in hospital-onset CDAD incidence rates or severity of CDAD (as measured by the number of colectomies per CDAD case per year and by the percentage of patients with CDAD who died during hospitalization) (data not shown). Thus, the high attributable mortality found in this study has important implications for patients: CDAD remains an important cause of patient death even in a CDAD-endemic setting.

Our study showed that CDAD had a delayed impact on death. In the matched-pairs analysis, the divergence in survival between CDAD cases-patients and controls did not begin until >60 days after hospital admission. Within 60 days of admission, survival was not significantly different between CDAD patients and controls, when all but 4 (1%) patients had been discharged from the hospital. This finding is consistent with those of 2 recent nested matched case–control studies in nonoutbreak settings, in which no significant excess deaths were reported after 30 days (*24*) or at discharge (*25*). Although CDAD can be acutely life-threatening, delayed death caused by CDAD may not be easily recognized as related to the initial CDAD episode. CDAD may contribute to a decline in patient function and overall illness over time, ultimately leading to death in many patients.

The results of the time-to-readmission and discharge location analyses further emphasize the negative impact of

Table 4. Cox proportional hazards model estimates of readmission and death of matched-pairs analysis, *Clostridium difficile*–associated disease (CDAD)*

Variable	CDAD case-patients, no. (%)	Controls, no. (%)	Hazard ratio (95% CI)
Readmitted within 180 d†	148 (51.0)	92 (31.7)	2.17 (1.59–2.95)
Deaths at 180 d‡	127 (36.0)	107 (30.3)	1.22 (0.92–1.61)
Deaths at 0–60 d‡	72 (20.4)	75 (21.2)	0.96 (0.54–1.70)
Deaths at 61–180 d‡	55 (15.6)	32 (9.1)	2.00 (1.47–2.72)

*CI, confidence interval.
†n = 290 matched pairs; 63 matched pairs were excluded because one or both patients in the pair died during the index hospital admission.
‡n = 353 matched pairs.

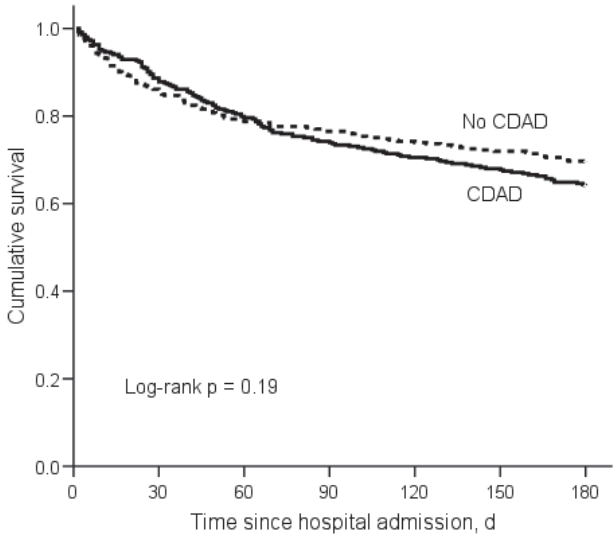


Figure 3. Kaplan-Meier survival estimates for matched pairs (n = 706). CDAD, *Clostridium difficile*–associated disease.

CDAD. CDAD patients were more than twice as likely to be readmitted to BJH within 180 days compared with controls. This finding is consistent with our prior findings that CDAD contributes to an increase in hospital costs extending out to at least 180 days (*26*). CDAD patients were also significantly more likely to be discharged to a long-term-care facility or outside hospital. Few data are available on the health of CDAD patients after hospital discharge, and future studies following CDAD patients as outpatients over an extended period are needed.

Data on the excess length of hospital stay attributable to CDAD are limited. Wilcox et al. found that CDAD patients stayed in the hospital, on average, 21.3 days longer than non-CDAD patients; however, the attributable length of stay was not calculated (*14*). O'Brien et al. reported that the mean increase in hospitalization among CDAD patients was 2.9 days (*27*). Kyne et al. calculated the attributable length of stay at 3.6 days (*11*), which was comparable to the attributable length of stay estimate found in our study (2.8 days).

Our study has several limitations, including the retrospective study design. Use of electronic data from the hospital's Medical Informatics database has limitations, although use of these data made analysis of such a large cohort feasible. Differences seen in observational studies may be due to unmeasured confounders. We attempted to address this issue by using 2 methods to control for confounding: multivariable regression analyses and propensity score matched-pairs analyses. As evident from the Kaplan-Meier mortality analyses, the matched-pairs population is a more homogeneous population than the cohort. This design allows more precise effect estimation because the association between CDAD and the propensity score variables among the study participants is eliminated. A strength of the multivariable regression analyses is the use of all available data in the cohort. In the propensity score matched-pairs analyses, 37 CDAD cases were excluded because of lack of a suitable control. Unmatched case-patients were more severely ill than matched case-patients, and their exclusion is a limitation of the propensity-score matched-pairs analyses. In the time-to-readmission analyses, we were unable to identify readmissions to hospitals other than our institution. Finally, surgical patients were excluded from these analyses. Because of this exclusion, the most severely ill CDAD patients requiring colectomies (n = 3) were not represented in the dataset. The absence of these patients, as well as the 37 unmatched case-patients, may have resulted in estimates of attributable length of stay and death that are biased low.

Data on attributable outcomes associated with CDAD are scarce. As previously mentioned, some data on attributable mortality and length of stay exist; however, these findings are limited by lack of adequate controls, small sample size, or outbreak settings. Our study provided detailed analysis on the effect of CDAD on time-to-readmission. Another key strength of this study is the combination of 2 analytical methods: Cox proportional hazards regression in the primary cohort and propensity score matched-pairs analysis. Mortality and time-to-readmission analyses, which were conducted in both the cohort and matched-pairs populations, had remarkably similar results. The results of this study suggest that endemic CDAD can lead to significantly poorer patient outcomes, including increased hospital length of stay, death, risk for admission to a long-term-care facility, and risk for hospital readmission. Even when the most severe CDAD cases are not considered, the detrimental effect of CDAD on patient health appears to extend beyond hospital discharge. Although prospective validation of these findings is needed, proper allocation of healthcare resources toward prevention of this infection is necessary to prevent further illness and death attributable to CDAD.

Acknowledgment

We thank Cherie Hill for technical assistance.

This work was supported by grants from the Centers for Disease Control and Prevention (UR8/CCU715087-06/1, 1U01C1000333-01) and the National Institutes of Health (nos. K12RR02324901-01, K24AI067794-01, K01AI065808-01).

Dr Dubberke is an assistant professor in the Division of Infectious Diseases, Department of Medicine, at Washington University School of Medicine. His research interests include *C. difficile*–associated disease, hospital epidemiology, and infections in transplant recipients.

References

1. McDonald LC, Owings M, Jernigan DB. *Clostridium difficile* infection in patients discharged from US short-stay hospitals, 1996–2003. Emerg Infect Dis. 2006;12:409–15.
2. Peterson CA, Calderon RL. Trends in enteric disease as a cause of death in the United States, 1989–1996. Am J Epidemiol. 2003;157:58–65. DOI: 10.1093/aje/kwf172
3. Gaynes R, Rimland D, Killum E, Lowery HK, Johnson TM, Killgore G, et al. Outbreak of *Clostridium difficile* infection in a long-term care facility: association with gatifloxacin use. Clin Infect Dis. 2004;38:640–5. DOI: 10.1086/381551
4. McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of *Clostridium difficile.* N Engl J Med. 2005;353:2433–41. DOI: 10.1056/NEJMoa051590
5. McEllistrem MC, Carman RJ, Gerding DN, Genheimer CW, Zheng L. A hospital outbreak of *Clostridium difficile* disease associated with isolates carrying binary toxin genes. Clin Infect Dis. 2005;40:265–72. DOI: 10.1086/427113
6. Muto CA, Pokrywka M, Shutt K, Mendelsohn AB, Nouri K, Posey K, et al. A large outbreak of *Clostridium difficile*–associated disease with an unexpected proportion of deaths and colectomies at a teaching hospital following increased fluoroquinolone use. Infect Control Hosp Epidemiol. 2005;26:273–80. DOI: 10.1086/502539
7. Pepin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, et al. *Clostridium difficile*–associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ. 2004;171:466–72. DOI: 10.1503/cmaj.1041104
8. Pepin J, Saheb N, Coulombe MA, Alary ME, Corriveau MP, Authier S, et al. Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*–associated diarrhea: a cohort study during an epidemic in Quebec. Clin Infect Dis. 2005;41:1254–60. DOI: 10.1086/496986
9. Pepin J, Valiquette L, Cossette B. Mortality attributable to nosocomial *Clostridium difficile*–associated disease during an epidemic caused by a hypervirulent strain in Quebec. CMAJ. 2005;173:1037–42. DOI: 10.1503/cmaj.050978
10. Dallal RM, Harbrecht BG, Boujoukas AJ, Sirio CA, Farkas LM, Lee KK, et al. Fulminant *Clostridium difficile*: an underappreciated and increasing cause of death and complications. Ann Surg. 2002;235:363–72. DOI: 10.1097/00000658-200203000-00008
11. Kyne L, Hamel MB, Polavaram R, Kelly CP. Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile.* Clin Infect Dis. 2002;34:346–53. DOI: 10.1086/338260
12. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, et al. A predominantly clonal multi-institutional outbreak of *Clostridium difficile*–associated diarrhea with high morbidity and mortality. N Engl J Med. 2005;353:2442–9. DOI: 10.1056/NEJMoa051639
13. Miller MA, Hyland M, Ofner-Agostini M, Gourdeau M, Ishak M. Morbidity, mortality, and healthcare burden of nosocomial *Clostridium difficile*–associated diarrhea in Canadian hospitals. Infect Control Hosp Epidemiol. 2002;23:137–40. DOI: 10.1086/502023
14. Wilcox MH, Cunniffe JG, Trundle C, Redpath C. Financial burden of hospital-acquired *Clostridium difficile* infection. J Hosp Infect. 1996;34:23–30. DOI: 10.1016/S0195-6701(96)90122-X
15. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. Crit Care Med. 1985;13:818–29. DOI: 10.1097/00003246-198510000-00009
16. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chronic Dis. 1987;40:373–83. DOI: 10.1016/0021-9681(87)90171-8
17. Deyo RA, Cherkin DC, Ciol MA. Adapting a clinical comorbidity index for use with ICD-9-CM administrative databases. J Clin Epidemiol. 1992;45:613–9. DOI: 10.1016/0895-4356(92)90133-8
18. Little RJA, Rubin DB. Statistical analysis with missing data. New York: Wiley; 2002.
19. Rosenbaum PR, Rubin DB. Constructing a control group using multivariable matched sampling methods that incorporate the propensity score. Am Stat. 1985;39:33–8. DOI: 10.2307/2683903
20. Kleinbaum DG. Survival analysis: a self-learning text. New York: Springer-Verlag; 1996.
21. Hubert B, Loo VG, Bourgault AM, Poirier L, Dascal A, Fortin E, et al. A portrait of the geographic dissemination of the *Clostridium difficile* North American pulsed-field type 1 strain and the epidemiology of *C. difficile*–associated disease in Quebec. Clin Infect Dis. 2007;44:238–44. DOI: 10.1086/510391
22. Wenzel RP. Perspective: attributable mortality–the promise of better antimicrobial therapy. J Infect Dis. 1998;178:917–9.
23. Dubberke ER, Reske KA, Noble-Wang J, Thompson A, Killgore G, Mayfield J, et al. Prevalence of *Clostridium difficile* environmental contamination and strain variability in multiple health care facilities. Am J Infect Control. 2007;35:315–8. DOI: 10.1016/j.ajic.2006.12.006
24. Kenneally C, Rosini JM, Skrupky LP, Doherty JA, Hollands JM, Martinez E, et al. An analysis of 30-day mortality for *Clostridium difficile*–associated disease in the ICU setting. Chest. 2007;132:418–24. DOI: 10.1378/chest.07-0202
25. Song X, McDonald LC, Bartlett JG, Speck K, Walsh B, Carroll K, et al. The rising economic impact of *Clostridium difficile*–associated disease (CDAD) in adult hospitalized patients. The Society for Healthcare Epidemiology in America 17th Annual Scientific Meeting, 2007 Apr 14–17. Abstract no. 183.
26. Dubberke ER, Reske K, Olsen M, McDonald L, Fraser V. Short and long-term attributable costs of *Clostridium difficile*–associated disease. Clin Infect Dis. 2008;46:497–504. DOI: 10.1086/526530
27. O'Brien JA, Lahue BJ, Caro JJ, Davidson DM. The emerging infectious challenge of C*lostridium difficile*–associated disease in Massachusetts hospitals: clinical and economic consequences. Infect Control Hosp Epidemiol. 2007;28:1219–27. DOI: 10.1086/522676

Address for correspondence: Erik R. Dubberke, Box 8051, 660 S Euclid, St. Louis, MO 63110, USA; email: edubberk@im.wustl.edu

Toxinotype V *Clostridium difficile* in Humans and Food Animals

Michael A. Jhung,* Angela D. Thompson,* George E. Killgore,* Walter E. Zukowski,† Glenn Songer,‡ Michael Warny,§ Stuart Johnson,†¶ Dale N. Gerding,†¶ L. Clifford McDonald,* and Brandi M. Limbago*

Clostridium difficile is a recognized pathogen in neonatal pigs and may contribute to enteritis in calves. Toxinotype V strains have been rare causes of human *C. difficile*–associated disease (CDAD). We examined toxinotype V in human disease, the genetic relationship of animal and human toxinotype V strains, and in vitro toxin production of these strains. From 2001 through 2006, 8 (1.3%) of 620 patient isolates were identified as toxinotype V; before 2001, 7 (<0.02%) of ≈6,000 isolates were identified as toxinotype V. Six (46.2%) of 13 case-patients for whom information was available had community-associated CDAD. Molecular characterization showed a high degree of similarity between human and animal toxinotype V isolates; all contained a 39-bp *tcdC* deletion and most produced binary toxin. Further study is needed to understand the epidemiology of CDAD caused by toxinotype V *C. difficile*, including the potential of foodborne transmission to humans.

Recent evidence suggests that the epidemiology of *Clostridium difficile*–associated disease (CDAD) is increasing in incidence and severity (*1*–*3*). These changes are due, at least in part, to the emergence of a more virulent *C. difficile* strain, designated NAP1 (based on its pulsed-field gel electrophoresis [PFGE] pattern), BI (by restriction endonuclease analysis [REA]), toxinotype III (by PCR characterization of the pathogenicity locus), and 027 (by PCR ribotyping) (*4*). However, the emergence of BI/NAP1/027 may not be solely responsible for changes in CDAD epidemiology, and the origin of this and other virulent strains is still largely unknown. *C. difficile* has also recently emerged as a pathogen or commensal in food animals such as neonatal pigs and beef and dairy calves (*5*–*7*); most of these animal isolates are toxigenic. Although several ribotypes have been identified in calves, the predominant ribotype in both calves and pigs is a toxinotype V strain (*8*,*9*). Moreover, recent reports suggest that *C. difficile* strains recognized as causes of human disease may contaminate retail meats (*10*).

To better understand whether food sources could be a source of infection for humans, we investigated recent and past human CDAD caused by toxinotype V *C. difficile* and compared isolates from these cases with those recovered from neonatal pigs and calves. We documented apparent changes in the frequency with which these toxinotype V strains cause human CDAD and compared the molecular characterization and toxin production of these strains with those of recent epidemic (i.e., BI/NAP1/027) and nonepidemic isolates.

Materials and Methods

Human Case Finding and Definitions

Case finding was performed by reviewing recent and past human isolates of interest from 2 sources. Cases were defined as patients with clinical isolates identified as toxinotype V by analysis of restriction fragment length polymorphisms (RFLPs) of toxin-encoding genes. First, we reviewed 620 *C. difficile* human isolates sent to the Centers for Disease Control and Prevention (CDC) from healthcare facilities and health departments in multiple states during hospital-associated outbreaks reported from 2001 through early 2007. Second, we reviewed a database of >6,000 isolates maintained by the Hines Veterans Affairs (VA) Hospital, representing CDAD reported from multiple healthcare

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Hines Veterans Affairs Hospital, Chicago, Illinois, USA; ‡University of Arizona, Tucson, Arizona, USA; §Acambis, Inc., Cambridge, Massachusetts, USA; and ¶Loyola University Chicago Stritch School of Medicine, Chicago

DOI: 10.3201/eid1407.071641

facilities from 1984 up to January 1, 2001. All toxinotype V isolates were obtained from patients with a diagnosis of CDAD based on clinical history (e.g., diarrhea) and a positive clinical laboratory test for *C. difficile* toxin (e.g., cytotoxin assay or enzyme immunoassay).

Isolates identified from the Hines VA Hospital database were designated "past," and isolates sent from hospitals to CDC from 2001 through early 2007 were designated "recent." Additional clinical information was obtained for recent case-patients from a standard reporting form completed by the original submitting institutions. The proportions of past and recent isolates identified as toxinotype V were compared with the χ^2 test results by using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA).

Case-patients were categorized with regard to likely place of acquisition according to recommendations developed by the *Clostridium difficile* Surveillance Working Group (*11*). Cases of CDAD were considered community-associated (CA-CDAD) if symptom onset (or positive *C. difficile* toxin test) occurred within ≤48 h after the patient was admitted to a healthcare facility, provided that it had been >12 weeks since the patient was last discharged from a healthcare facility. Cases of CDAD were considered healthcare facility–associated CDAD (HCFA-CDAD) if the patient had symptom onset >48 h after admission to a healthcare facility or was discharged from a healthcare facility within the previous 4 weeks. Cases were classified as indeterminate if symptom onset was within ≤48 h of a patient's admission to a healthcare facility and 4–12 weeks since discharge from a previous admission. Case-patients were considered exposed to antimicrobial agents if the patient received a dose of any antimicrobial agent within the 30 days before symptom onset.

Laboratory Methods

C. difficile isolates from humans and animals with clinical disease were obtained from diagnostic laboratories as previously described (*4*,*8*). Swine isolates were obtained from neonatal pigs with CDAD in North Carolina, Iowa, Texas, Utah, Ohio, and Arizona from 1999 to 2005. Bovine strains were isolated from January 1, 2003, through 2005, mainly from diarrheic Holstein calves 1 day to 6 weeks of age, originating in southern California, Arizona, New Mexico, Nevada, Texas, and Utah and maintained in prefeedlot housing in calf ranches in Arizona. Domestic animal isolates were selected for study on the basis of host of origin, date of isolation, and geographic origin, all of which were independent. All food animal and human isolates were typed by PFGE with *Sma*I digestion as previously described (*12*,*13*) and analyzed with BioNumerics software version 4.01 (Applied Maths, Austin, TX, USA). Repeat PFGE analysis was performed with *Eag*I-digested DNA if human–food animal isolate pairs were indistinguishable when subjected to PFGE after digestion with *Sma*I or when *Sma*I digestion yielded too few bands for analysis. REA typing was performed, and patterns were compared as previously described (*12*). RFLP analysis of PCR fragments A3 and B1, from within *tcdA* and *tcdB*, respectively, was performed as previously described to determine toxinotypes (*14*).

PCR was used to detect *cdtB*, one of the genes encoding binary toxin. Deletions in *tcdC* were detected by PCR with primers tcdc1 and tcdc2, as described (*15*).

A subset of toxinotype V animal isolates (7 bovine, 7 porcine) and 7 recent human isolates were selected for toxin quantification. Production of toxins A and B was measured by ELISA as previously described (*1*). Toxin production was measured at 24 h, 48 h, and 72 h, and cell growth at 24 h and 48 h. Cell growth and in vitro toxin A and B production were compared between combined animal and human isolates of toxinotype V to human epidemic strain isolates (i.e., BI/NAP1/027; toxinotype III), and recent nonepidemic human strains (toxinotype 0) (*1*). Cell growth was compared by using the Student *t* test; the Mann-Whitney test was used to compare toxin production because toxin production values were not normally distributed.

Fourteen human (7 recent, 7 past) and 16 animal (8 bovine, 8 porcine) toxinotype V isolates were randomly selected for antimicrobial drug susceptibility testing. Susceptibility to clindamycin, levofloxacin, moxifloxacin, and gatifloxacin was determined by using E-test strips (AB Biodisk, Piscataway, NJ, USA) on Brucella agar plates with 5% sheep blood (Remel, Lenexa, KS, USA). Results were interpreted according to Clinical and Laboratory Standards Institute standard criteria (*16*). However, because no breakpoints have been established for levofloxacin and gatifloxacin, moxifloxacin breakpoints were used for interpretation of these results.

Results

Human Cases

Seven past cases of human infection with toxinotype V *C. difficile* were identified among the ≈6,000 human isolates in the Hines VA database; these cases occurred over 11 years before 2001. Eight additional recent cases were identified among the 620 human isolates sent to CDC from multiple states during 2001 through April 2006. The difference in proportions of past (<0.2%) and recent (1.3%) isolates that were toxinotype V was statistically significant ($p<0.001$). Three (38%) of 8 recent cases were CA-CDAD, 7 (88%) of such patients were exposed to antimicrobial agents, and 1 (13%) patient died from complications attributed to CDAD (Table 1). Four recent case-patients (50%) were male, and the median age was 71 years. Among patients for whom records were available,

Table 1. Clinical epidemiology of human toxinotype V *Clostridium difficile* cases, 1989–2006*

Origin	Location	Sex	Age, y	Antimicrobial agents†	Etiology	Diagnosis date	Disposition
1 R	Pennsylvania	F	75	Yes	HCFA	2001 May 2	Died
2 R	Illinois	M	54	Yes	CA	2003 Jul 24	Sent home
3 R	Iowa	F	71	Yes	HCFA	2004 Jul 29	Sent home
4 R	Texas	M	56	Yes	HCFA	2004 Nov 5	Sent home
5 R	Connecticut	F	85	Yes	HCFA	2004 Nov 21	Died
6 R	Georgia	M	72	Yes	CA	2005 Feb 8	Sent home
7 R	Connecticut	F	78	No	IND	2005 Jun 18	Sent home
8 R	Massachusetts	M	51	Yes	CA	2006 Jan 20	Died‡
1 P	Minnesota	M	NA	Yes	HCFA	1989 Apr 26	Unknown
2 P	Arizona	NA	NA	Unknown	Unknown	1991	Unknown
3 P	Illinois	M	71	Unknown	CA	1995 Mar 28	Unknown
4 P	Illinois	M	71	Yes	HCFA	1995 Apr 5	Unknown
5 P	Belgium	NA	NA	Unknown	Unknown	<1996	Unknown
6 P	Illinois	M	73	Yes	CA	1999 Sep 21	Sent home
7 P	Illinois	M	60	Yes	CA	1999 Nov 24	Sent home

*R, recent; P, past; HCFA, healthcare facility–associated; CA, community-associated; IND, indeterminate (patient with symptom onset ≤48 h of admission to healthcare facility and 4–12 wks since discharge from previous admission); NA, not available.
†History of antimicrobial drug use within 30 d before *C. difficile*–associated disease (CDAD) diagnosis.
‡Death attributed to CDAD.

3 (60%) of 5 past cases were judged to be CA-CDAD and 4 of 4 past cases were in persons exposed to antimicrobial agents.

Laboratory Results

All 8 recent human isolates had a 39-bp deletion in *tcdC,* and 6 (75%) of 8 were binary toxin positive. All 7 past human isolates also had a 39-bp deletion in *tcdC,* and 7 (100%) of 7 were binary toxin positive. Thirty-three toxinotype V animal isolates were obtained; they displayed a variety of PFGE patterns (Figure 1). All, however, were binary toxin positive and had a 39-bp deletion in *tcdC*. Three animal–human isolate groups had indistinguishable PFGE patterns (100% similarity) when digestion was performed with the *Sma*I enzyme. The first group contained 1 human isolate (REA subtype BK1) that was indistinguishable by PFGE (NAP7) from 1 porcine isolate (REA subtype BK13). The second group consisted of 5 human and 2 porcine isolates, all of which were designated NAP7 by PFGE, although REA demonstrated 4 different subtypes. The third group contained 1 human isolate and 2 porcine isolates, which were indistinguishable by PFGE and REA (NAP8 and BK6). The 9 isolates in groups 1 and 2 were only 80% similar when digestion was performed with *Eag*I. However, the isolates in the third group were 95% similar, and 1 porcine isolate was indistinguishable (100% similarity) from 1 human isolate, even after digestion with *Eag*I.

Median toxin A and B production in the 21 toxinotype V isolates analyzed (7 bovine, 7 porcine, 7 of 8 recent human) was greater than that by nonepidemic toxinotype 0 isolates but less than that by epidemic toxinotype III isolates at all time points measured (Figure 2). The mean absorbance measurements at 600 nm, representing cell density, were measured at 24 h and 48 h and were not significantly different for toxinotype V isolates (1.56 and 1.06, respectively) than for toxinotype 0 isolates (1.77 and 1.39) or toxinotype III isolates (2.07 and 1.64).

Antimicrobial drug susceptibility testing was performed on 14 of 15 human and 16 of 33 animal toxinotype V isolates (Table 2). Resistance rates were similar overall in human and animal toxinotype V isolates except that more bovine isolates (88%) were susceptible to clindamycin than were porcine (0%, $p<0.01$) or human isolates (9%, $p<0.01$). All human and animal toxinotype V isolates (multiple strains by REA and PFGE) were susceptible to gatifloxacin and moxifloxacin, which differed markedly from human toxinotype III (BI/NAP1/027) and toxinotype 0 isolates (multiple strains by REA and PFGE).

Discussion

In a review of recent and past isolates, we identified several human cases of CDAD caused by toxinotype V strains of *C. difficile*, which has been reported as a cause of epidemic disease in neonatal pigs and colonization in calves during the past decade (*9*,*17*,*18*). Moreover, different rates of occurrence in these temporally divergent populations suggest that toxinotype V may be an increasing cause of human CDAD, relative to other strains. The toxinotype V animal isolates included in our study have been previously identified as PCR ribotype 078, the most prevalent ribotype among calves and pigs, accounting for 94% (bovine) and 83% (swine) of isolates tested from multiple geographic regions (*8*). Food animal isolates we tested shared a high degree of similarity with human isolates, with 2 instances of animal–human isolate pairs appearing indistinguishable by REA or PFGE subtyping. In addition, all animal and human isolates displayed 39-bp deletions in *tcdC*, and most (45/47; 96%) were binary toxin positive.

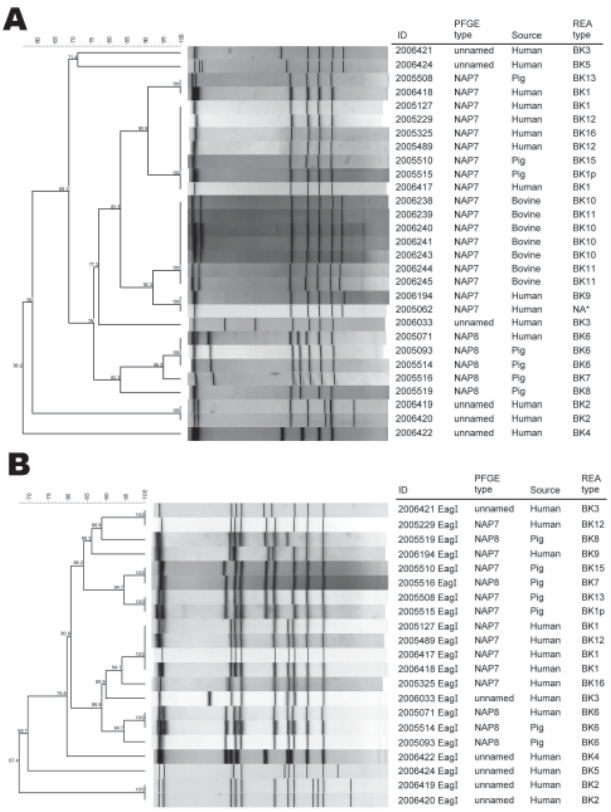


Figure 1. A). Dendrogram analysis of toxinotype V *Clostridium difficile* human and animal isolates using pulsed field gel electrophoresis (PFGE); *Sma*I restriction digest. Three animal-human isolate groups had indistinguishable PFGE patterns (2 NAP7 and 1 NAP8 group). Three of the NAP8 isolates (2005071, 2005093, 2005524) had identical REA types (BK6) as well. *PCR type unavailable. B) Dendrogram analysis using PFGE; *Eag*I restriction digest. One human–pig pair (2005071 and 2005514) had identical PFGE patterns by both *Eag*I and *Sma*I as well as identical REA patterns (BK6). Digestion of bovine isolates with *Eag*I yielded results that were not interpretable and were not included in this figure.

Although *C. difficile* is recognized as a cause of disease in several animal species (*19–22*), little investigation has been conducted on the potential for interspecies transmission of *C. difficile* to humans. Previous studies have suggested the possibility of *C. difficile* transmission between humans and domestic pets (*23*,*24*), but no interspecies transmission has been documented, and few studies have examined the possible link between CDAD in food animals and humans. Identification of the same variant toxinotype strain as responsible for both human and animal disease in our study suggests at least 3 possible causes for human toxinotype V CDAD: 1) exposure of humans and animals to a common environmental source of *C. difficile*, 2) human disease caused by transmission by means of direct or indirect (e.g., through contaminated produce, water, or the environment) contact with infected live animals, and 3) human disease linked to consumption of products from food-producing animals. Both the genetic similarity of the human and animal isolates in our study and the apparent increasing importance of toxinotype V isolates in human CDAD after their emergence in animals may suggest foodborne or other forms of animal-to-human transmission.

In contrast to HCFA-CDAD, where patient-to-patient transmission of *C. difficil*e is more likely, animal contact is a more plausible means of transmission for CA-CDAD. Our results suggest that toxinotype V *C. difficile* may be a relatively common cause of community-associated disease. Our results suggest that toxinotype V *C. difficile* may be a relatively common cause of community-associated disease. Despite evidence that only 20% of all human CDAD cases are community-associated (*25*,*26*), 6 (46%) of 13 human toxinotype V cases in our study were identified as CA-CDAD. The high prevalence of CA-CDAD among toxinotype V cases we found is consistent with other studies that have identified variant toxinotypes more frequently in CA-CDAD than in HCFA-CDAD (*27*,*28*).

Toxinotype V strains may also be increasing as a cause of human CDAD since the emergence or recognition of epidemic toxinotype V disease in animals. In the past, reported frequencies of human strains with variant toxinotypes ranged from 6.4% to 13.4% of all *C. difficile* isolates collected (*29–33*), and toxinotype V strains contributed few cases to these frequency studies. However, a recent preliminary report from an Italian hospital indicated an upsurge in the proportion of binary toxin–positive *C. difficile* strains responsible for healthcare-associated disease in 2002 and 2003; most of these strains were toxinotype V (*34*). Toxinotype V appears to be an important cause of CDAD in food-producing pigs in parts of Europe, just as it is in North America (*35*).

The epidemiology of human CDAD has been affected by recent increases in the incidence and severity of disease. These changes have been largely attributed to the emergence of the BI/NAP1/027 *C. difficile* strain which, like the toxinotype V strains described here, is a toxin gene variant (i.e., toxinotype III) with an 18-bp deletion in *tcdC* (rather than the 39-bp deletion observed in toxinotype V strains) and with genes that encode binary toxin (*4*). In addition to this 18-bp deletion, and perhaps more importantly, BI/NAP1/027 has an upstream single nucleotide deletion at nucleotide position 117 (Δ117), leading to a reading frameshift and early termination of protein translation (*27*).

Current literature suggests that this considerable truncation of TcdC may impair its negative regulatory function and contribute to the increased toxin production observed in BI/NAP1/027 strains (*27*,*36*). Molecular analysis of toxinotype V *C. difficile* has demonstrated a similarly truncated TcdC (61 aa compared with 65 aa in BI/NAP1/027 strains and 232 aa in wild-type TcdC) (*15*), which may imply

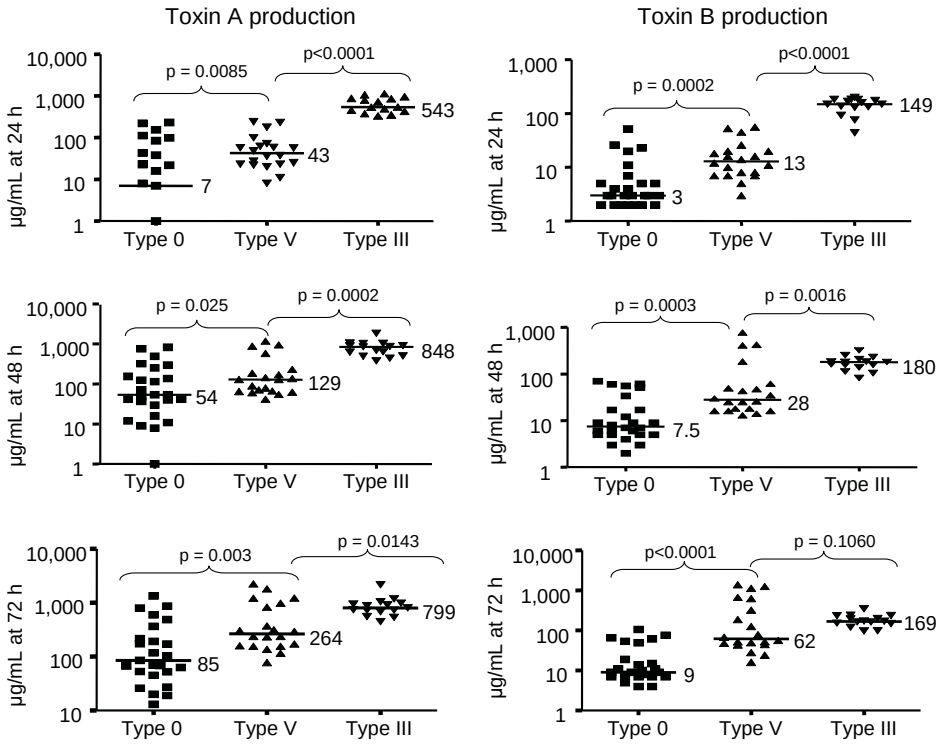


Figure 2. In vitro toxin production of toxinotype V *Clostridium difficile* isolates compared with epidemic toxinotype III and nonepidemic toxinotype 0 strains. Toxin A and Toxin B concentrations in micrograms per milliliter at 24, 48, and 72 h are shown for 25 toxinotype 0 isolates, 21 toxinotype V isolates (7 human; 14 animal), and 15 toxinotype III isolates. Horizontal lines indicate median values for each group and the p values are shown for comparison of the median toxin levels of toxinotype V isolates with toxinotype 0 and toxinotype III isolates.

hypervirulence for this strain as well. In contrast, isolates most commonly found in US hospitals before 2001 were toxinotype 0, had no polymorphisms in *tcdC*, and were binary toxin negative (*37*). Some of the increased virulence of BI/NAP1/027 may be due to its documented increased toxin A and B production in vitro (*1*). Although we did not find toxin production in toxinotype V isolates similar to BI/NAP1/027 levels, they did produce more toxin than nonepidemic toxinotype 0 isolates at all time points. Furthermore, the range of toxin A and B levels in our toxinotype V isolates was wide, and a minority produced toxin at similar or greater levels than BI/NAP1/027 strains.

This study is subject to the following limitations. First, the number of toxinotype V isolates examined was small and may not be wholly representative of this strain as it manifests in human or animal disease. Furthermore, recent isolates were collected from institutions that reported healthcare-associated outbreaks of CDAD, and clinical information describing patients from whom specimens were obtained may therefore overestimate disease severity. Moreover, since recent isolates were obtained from healthcare facilities that were experiencing CDAD outbreaks, they may represent a different population of patients than the past isolates, which were obtained from a variety of sources, some of which were ongoing clinical surveillance projects and some of which were outbreak investigations. The 2 source populations, however, can be considered reasonably similar in that both represent primarily hospitalized patients. If, however, the recent database contains a substantially greater proportion of outbreak-related isolates than the past collection, this would only strengthen the evidence for the recent emergence of toxinotype V CDAD. Since outbreaks of CDAD are largely associated with the epidemic, toxinotype III strain of *C. difficile* (*4*), relatively few toxinotype V isolates should be present in a recent database composed of outbreak-related isolates. The increased

Table 2. Antimicrobial drug susceptibility of toxinotype V *Clostridium difficile* isolates, 1989–2006*

		Clindamycin			Levofloxacin†			Moxifloxacin		Gatifloxacin†	
Source	N	S	I/R	Range	S	I/R	Range	S	Range	S	Range
Human	14	2 (14)	12 (86)	2–>256	6 (43)	8 (57)	2–>32	14 (100)	0.5–1	14 (100)	0.5–1
Recent	7	2 (29)	5 (71)	2–>256	0	7 (100)	>32	7 (100)	0.5	7 (100)	0.5–1
Past	7	0	7 (100)	4–>256	7 (100)	0	2–4	7 (100)	0.5–1	7 (100)	0.5–1
Porcine	8	0	8 (100)	4––>256	2 (25)	6 (75)	2–>32	8 (100)	0.5–1	8 (100)	0.5–1
Bovine	8	7 (88)	1 (12)	1–4	8 (100)	0	2–4	8 (100)	0.5	8 (100)	0.5

*No. (%) *Clostidium difficile* isolates shown in each interpretive category. S, susceptible; I/R, intermediate or resistant.
†Using moxifloxacin interpretive criteria.

prevalence of toxinotype V in recent isolates compared with past ones may therefore represent an underestimate of the true prevalence of toxinotype V *C. difficile*.

Additionally, little is known about the types of *C. difficile* that cause disease in animals, which makes it impossible to determine whether the current toxinotype V strains are new or simply newly recognized. Finally, information about human cases is limited, particularly with respect to possible routes for community acquisition of disease; thus, evidence upon which to base conclusions regarding interspecies transmission is limited.

Although relatively common in animal CDAD, toxinotype V is currently an uncommon cause of human illness, which may occur more frequently among persons without traditional risk factors associated with CDAD, such as recent exposure to a healthcare setting. In vitro toxin production results from our limited sample suggest that toxinotype V strains have the potential to cause increased severity of human disease, although further studies are needed to corroborate this association. Although they share similar clinical features, evidence suggests that the predominant strains causing CDAD in humans and different animal species are distinct (*8*,*38*). Nonetheless, our finding of similarity between relatively widespread animal strains of *C. difficile* and strains responsible for occasional human disease raises the possibility of interspecies transmission. Further studies are needed to understand the etiology of CDAD caused by toxinotype V *C. difficile* and the mechanisms of transmission between animals and humans, including the role of the food supply.

Dr Jhung is a medical epidemiologist in the Division of Healthcare Quality Promotion at CDC. His primary interests include the epidemiology of healthcare-associated infections, outbreak investigations, infection control in resource-limited settings, and the public health response to disasters and emergencies.

References

1. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 2005;366:1079–84. DOI: 10.1016/S0140-6736(05)67420-X
2. Kuijper EJ, Coignard B, Tull P. Emergence of *Clostridium difficile*–associated disease in North America and Europe. Clin Microbiol Infect. 2006;12(Suppl 6):2–18. DOI: 10.1111/j.1469-0691.2006.01580.x
3. Sunenshine RH, McDonald LC. *Clostridium difficile*–associated disease: new challenges from an established pathogen. Cleve Clin J Med. 2006;73:187–97.
4. McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of *Clostridium difficile.* N Engl J Med. 2005;353:2433–41. DOI: 10.1056/NEJMoa051590
5. Songer JG, Post KW, Larson DJ, Jost BH, Glock RD. Enteric infection of neonatal swine with *Clostridium difficile.* Journal of Swine Health and Production. 2000;8:185–9.
6. Songer JG, Post KW, Larson DJ, Jost BH, Glock RD. *Clostridium difficile* as a cause of porcine neonatal enteritis. Birmingham (AL): American Association of Veterinary Laboratory Diagnosticians; 2000.
7. Post KW, Songer JG, Jost BH, Glock RD, Holtcamp A. The emergence of *Clostridium difficile* as a cause of porcine neonatal enteritis. Nashville (TN): American Association of Swine Veterinarians; 2001.
8. Keel K, Brazier JS, Post KW, Weese S, Songer JG. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J Clin Microbiol. 2007;45:1963–4. DOI: 10.1128/JCM.00224-07
9. Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS. *Clostridium difficile* in retail ground meat, Canada. Emerg Infect Dis. 2007;13:485–7.
10. Rodriguez-Palacios A, Staempfli HR, Duffield T, Peregrine AS, Trotz-Williams LA, Arroyo LG, et al. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg Infect Dis. 2006;12:1730–6.
11. McDonald LC, Coignard B, Dubberke E, Song X, Horan T, Kutty PK. Recommendations for surveillance of *Clostridium difficile*–associated disease. Infect Control Hosp Epidemiol. 2007;28:140–5. DOI: 10.1086/511798
12. Clabots CR, Johnson S, Bettin KM, Mathie PA, Mulligan ME, Schaberg DR, et al. Development of a rapid and efficient restriction endonuclease analysis typing system for *Clostridium difficile* and correlation with other typing systems. J Clin Microbiol. 1993;31:1870–5.
13. Klaassen CH, van Haren HA, Horrevorts AM. Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. J Clin Microbiol. 2002;40:101–4. DOI: 10.1128/JCM.40.1.101-104.2002
14. Rupnik M, Avesani V, Janc M, von Eichel-Streiber C, Delmee M. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J Clin Microbiol. 1998;36:2240–7.
15. Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J Clin Microbiol. 2002;40:3470–5. DOI: 10.1128/JCM.40.9.3470-3475.2002
16. National Committee for Clinical Laboratory Standards. Methods of antimicrobial susceptibility testing of anaerobic bacteria. 6th ed. Approved standard M11–A6. Villanova (PA): The Committee; 2004.
17. Songer JG, Anderson MA. *Clostridium difficile*: an important pathogen of food animals. Anaerobe. 2006;12:1–4. DOI: 10.1016/j.anaerobe.2005.09.001
18. Songer JG. The emergence of *Clostridium difficile* as a pathogen of food animals. Anim Health Res Rev. 2004;5:321–6. DOI: 10.1079/AHR200492
19. Frazier KS, Herron AJ, Hines ME II, Gaskin JM, Altman NH. Diagnosis of enteritis and enterotoxemia due to *Clostridium difficile* in captive ostriches (*Struthio camelus*). J Vet Diagn Invest. 1993;5:623–5.
20. Marks SL, Kather EJ, Kass PH, Melli AC. Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs. J Vet Intern Med. 2002;16:533–40. DOI: 10.1892/0891-6640(2002)016<0533:GAPCOP>2.3.CO;2
21. Weese JS, Weese HE, Bourdeau TL, Staempfli HR. Suspected *Clostridium difficile*–associated diarrhea in two cats. J Am Vet Med Assoc. 2001;218:1436–9, 1421.
22. Weese JS, Staempfli HR, Prescott JF. A prospective study of the roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in equine diarrhoea. Equine Vet J. 2001;33:403–9. DOI: 10.2746/042516401776249534

23. O'Neill G, Adams JE, Bowman RA, Riley TV. A molecular characterization of *Clostridium difficile* isolates from humans, animals and their environments. Epidemiol Infect. 1993;111:257–64.
24. Lefebvre SL, Arroyo LG, Weese JS. Epidemic *Clostridium difficile* strain in hospital visitation dog. Emerg Infect Dis. 2006;12:1036–7.
25. Kutty PK, Benoit SR, Woods CW, Sena AC, Naggie S, Frederick J, et al. Assessment of *Clostridium difficile*–associated disease surveillance definitions, North Carolina, 2005. Infect Control Hosp Epidemiol. 2008;29:197–202. DOI: 10.1086/528813
26. Noren T, Akerlund T, Back E, Sjoberg L, Persson I, Alriksson I, et al. Molecular epidemiology of hospital-associated and community-acquired *Clostridium difficile* infection in a Swedish county. J Clin Microbiol. 2004;42:3635–43. DOI: 10.1128/JCM.42.8.3635-3643.2004
27. MacCannell DR, Louie TJ, Gregson DB, Laverdiere M, Labbe AC, Laing F, et al. Molecular analysis of *Clostridium difficile* PCR ribotype 027 isolates from Eastern and Western Canada. J Clin Microbiol. 2006;44:2147–52. DOI: 10.1128/JCM.02563-05
28. Limbago B, Long CM, Thompson AD, Killgore GE, Hannett G, Havill N, et al. Isolation and characterization of *Clostridium difficile* responsible for community-associated disease. International Meeting of the American Society for Microbiology; 2007 May 21–25; Toronto, Ontario, Canada.
29. Barbut F, Gariazzo B, Bonne L, Lalande V, Burghoffer B, Luiuz R, et al. Clinical features of *Clostridium difficile*–associated infections and molecular characterization of strains: results of a retrospective study, 2000–2004. Infect Control Hosp Epidemiol. 2007;28:131–9. DOI: 10.1086/511794
30. Geric B, Rupnik M, Gerding DN, Grabnar M, Johnson S. Distribution of *Clostridium difficile* variant toxinotypes and strains with binary toxin genes among clinical isolates in an American hospital. J Med Microbiol. 2004;53:887–94. DOI: 10.1099/jmm.0.45610-0
31. Rupnik M, Kato N, Grabnar M, Kato H. New types of toxin A-negative, toxin B-positive strains among *Clostridium difficile* isolates from Asia. J Clin Microbiol. 2003;41:1118–25. DOI: 10.1128/JCM.41.3.1118-1125.2003
32. Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile.* FEMS Microbiol Lett. 2000;186:307–12. DOI: 10.1111/j.1574-6968.2000.tb09122.x
33. Pituch H, Kreft D, Obuch-Woszczatynski P, Wultanska D, Meisel-Mikolajczyk F, Luczak M, et al. Clonal spread of a *Clostridium difficile* strain with a complete set of toxin A, toxin B, and binary toxin genes among Polish patients with *Clostridium difficile*–associated diarrhea. J Clin Microbiol. 2005;43:472–5. DOI: 10.1128/JCM.43.1.472-475.2005
34. Buttrini M, Spigaglia P, Somenzi P, Zerbini L, Dettori G, Chezzi C, et al. Epidemiology of *Clostridium difficile* strains with binary-toxin genes among clinical isolates in an Italian hospital. 2nd International *Clostridium difficile* Symposium; 2007 June 6–9; Maribor, Slovenia.
35. Pirs T, Avbersek J, Ocepek M, Rupnik M. Isolation of *Clostridium difficile* from food animals in Slovenia. 2nd International *Clostridium difficile* Symposium; 2007 June 6–9; Maribor, Slovenia.
36. Curry SR, Marsh JW, Muto CA, O'Leary MM, Pasculle AW, Harrison LH. *tcdC* genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile.* J Clin Microbiol. 2007;45:215–21. DOI: 10.1128/JCM.01599-06
37. McFarland LV, Beneda HW, Clarridge JE, Raugi GJ. Implications of the changing face of *Clostridium difficile* disease for health care practitioners. Am J Infect Control. 2007;35:237–53. DOI: 10.1016/j.ajic.2006.06.004
38. Arroyo LG, Kruth SA, Willey BM, Staempfli HR, Low DE, Weese JS. PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. J Med Microbiol. 2005;54:163–6. DOI: 10.1099/jmm.0.45805-0

Address for correspondence: Michael A. Jhung, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop A31, Atlanta, GA 30333, USA; email: mjhung@cdc.gov

etymologia

Sapovirus

[Sap′ o-vi″ rəs]

Sapovirus, formerly Sapporo-like virus after Sapporo, Japan, where first recognized during an outbreak in an orphanage in 1977. A genus of viruses of the family Caliciviridae, they cause self-limited, acute foodborne gastroenteritis. Morphologically similar viruses were detected in a subsequent series of outbreaks in the same institution between 1977 and 1982. Sapoviruses play an important role in outbreaks of gastroenteritis in infants and have recently been found to infect adults.

Source: Dorland's illustrated medical dictionary, 31st ed. Philadelphia: Saunders/Elsevier; 2007; Chiba S, Nakata S, Numata-Kinoshita K, Honma S. Sapporo virus: history and recent findings. J Infect Dis. 2000;181(Suppl 2):S303–8.

Alcaligenes xylosoxidans Bloodstream Infections in Outpatient Oncology Office

Moon J. Kim,* Elizabeth Bancroft,* Eleanor Lehnkering,* Rodney M. Donlan,† and Laurene Mascola*

CME ACTIVITY

Medscape, LLC is pleased to provide online continuing medical education (CME) for this journal article, allowing clinicians the opportunity to earn CME credit. Medscape, LLC is accredited by the Accreditation Council for Continuing Medical Education (ACCME) to provide CME for physicians. Medscape, LLC designates this educational activity for a maximum of 0.5 *AMA PRA Category 1 Credits*™. Physicians should only claim credit commensurate with the extent of their participation in the activity. All other clinicians completing this activity will be issued a certificate of participation. To participate in this journal CME activity: (1) review the learning objectives and author disclosures; (2) study the education content; (3) take the post-test and/or complete the evaluation at **http://www.medscape.com/cme/eid**; (4) view/print certificate.

Learning Objectives

Upon completion of this activity, participants will be able to:

- Identify properties of *Alcaligenes xylosoxidans*.
- Describe the clinical presentation of *A. xylosoxidans* infection in the current study.
- Specify risk factors for infection with *A. xylosoxidans*.
- Identify the primary source of infection with *A. xylosoxidans* in the current study.

Editor

D. Peter Drotman, MD, Editor-in-Chief, Emerging Infectious Diseases. *Disclosure: D. Peter Drotman, MD, has disclosed no relevant financial relationships.*

CME Author

Charles P. Vega, MD, Associate Professor; Residency Director, Department of Family Medicine, University of California, Irvine, California, USA. *Disclosure: Charles P. Vega, MD, has disclosed that he has served as an advisor or consultant to Novartis, Inc.*

Authors

Disclosures: ***Moon Kim, MD, MPH; Elizabeth Bancroft, MD, SM; Eleanor Lehnkering, MS;*** *and* ***Rodney M. Donlan, PhD,*** *have disclosed no relevant financial relationships.* ***Laurene Mascola, MD, MPH,*** *has disclosed that she has served has an advisor or consultant to Merck and MedImmune. Dr. Mascola has also disclosed that she has served as a speaker for Merck.*

In 2002, we investigated a cluster of patients with *Alcaligenes xylosoxidans* bloodstream infections by conducting a matched case–control study and a prospective study. Pulsed-field gel electrophoresis (PFGE) was performed on blood culture isolates, and 1 explanted central venous catheter (CVC) was tested for biofilm. We identified 12 cases of *A. xylosoxidans* bloodstream infection. Case-patients were more likely than controls to have had a CVC (7/7 [100%] vs 4/47 [8.7%], respectively; p<0.0001). Ten case isolates were indistinguishable by PFGE analysis, and *A. xylosoxidans* biofilm from the CVC matched the outbreak strain. We observed multiple breaches in infection control, which may have caused contamination of multidose vials used to flush the CVCs. Our study links *A. xylosoxidans* with CVC biofilm and highlights areas for regulation and oversight in outpatient settings.

*Los Angeles County Department of Public Health, Los Angeles, California, USA; and †Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: 10.3201/eid1407.070894

Alcaligenes xylosoxidans, also known as *Achromobacter xylosoxidans*, is a gram-negative, water-borne organism that causes healthcare-associated infections (*1*–*7*) and bacteremia in immunocompromised patients with indwelling catheters (*6*–*11*); it can also contaminate liquids (*2*,*5*,*12*–*14*). *A. xylosoxidans* is found in soil and water and grows in saline (*15*,*16*). On January 16, 2002, the Acute Communicable Disease Control (ACDC) program of the Los Angeles County Department of Public Health was notified by a local hospital epidemiologist about a cluster of patients with *A. xylosoxidans* bloodstream infections (*17*). The patients had been admitted to Hospital A over a period of 2 months.

To confirm the presence of an outbreak, ACDC conducted a telephone survey, which asked the microbiology laboratories of Hospital A and 4 surrounding hospitals for a list of all patients who had had positive blood cultures for *A. xylosoxidans* in the past 3 months. One laboratory identified 3 such patients, and Hospital A laboratory identified 7; all 9 patients (1 case-patient had positive blood

cultures reported from both laboratories) were associated with a single outpatient oncology office, Office B. The other 3 hospitals reported that they had not identified any *A. xylosoxidans* bloodstream infections in the past 3 months. To identify the source of the outbreak and risk factors for infection and to implement control measures, ACDC then initiated an outbreak investigation.

Methods

The outbreak investigation focused on Office B. To identify all patients associated with Office B who had had a positive *A. xylosoxidans* culture, we requested a review of medical records and laboratory reports.

Matched Case–Control Study

A matched case–control study was performed to determine risk factors for infection. A case-patient was defined as a patient of Office B who had a positive *A. xylosoxidans* blood culture from November 2001 through January 2002. Controls were defined as patients who had no symptoms or signs of bloodstream infection (fever, chills, rigors, myalgias, nausea, vomiting, weakness, or hypotension). For each case-patient, 5–7 controls were randomly selected and matched by the closest date of their visits to Office B to the case-patient's date of visit. Variables included age, sex, underlying diagnosis, intravenous medications received, peripheral white blood cell counts, presence and type of central venous catheter (CVC), clinic visits, hospitalization dates, symptoms, and types of invasive procedures. Data were collected on standardized forms and analyzed by using Epi Info 2000 version 1.1.2 (Centers for Disease Control and Prevention [CDC], Atlanta, GA, USA); odds ratios were used to estimate risk, *t* tests were performed for continuous variables, and $p<0.05$ indicated statistical significance.

Prospective Cohort Study

To identify possible *A. xylosoxidans* bloodstream infection, ACDC conducted prospective blood culture surveillance. On February 15, 2002, all patients with a CVC who had visited Office B since November 2001 were sent a letter requesting them to have a culture performed on blood drawn through the CVC. CVCs were removed from those whose culture results were positive.

Environmental Investigation

On January 17, 2002, numerous open containers (multidose 30-mL vials of heparin; 100-mL and 150-mL bottles of saline; and containers of alcohol, hydrogen peroxide, betadine, and iodine) were collected and sent to the Los Angeles County Public Health Laboratory for analysis. On February 15, 2002, environmental samples and swabs were collected for culture from work surfaces (e.g., countertops, sinks, hoods, kitchens) and from tap water and hands of healthcare workers who accessed CVCs, collected blood, prepared flushes, or administered chemotherapy.

Infection Control

During January and February 2002, we made several site visits to Office B to observe procedures, review medical records, and interview the office staff. Specifically, we observed procedures for preparation and administration of intravenous medications and asked office staff about level of technical training, experience, and license status.

Molecular Studies

Blood isolates of *A. xylosoxidans* from case-patients were obtained from Hospital A's laboratory. For comparison, all *A. xylosoxidans* isolated from patients from Los Angeles County in the past 6 months were obtained from a large local reference laboratory. Pulsed-field gel electrophoresis (PFGE) was performed at the Los Angeles County Public Health Laboratory by using standard methods (*18*) for *Salmonella* spp. with the exception that isolates were digested with *Xba*I and *Spe*I. PFGE pattern comparisons were performed visually by using criteria established by Tenover et al. (*19*).

Examination of CVC for Biofilm

A CVC (PASport, a single-lumen, 6 French catheter with an under-the-skin titanium port; SIMS Deltec, Inc., St. Paul, MN, USA) was surgically removed from an asymptomatic patient identified in the prospective cohort study. Aseptic methods were used. The distal lumen opening was clamped with a sterile hemostat to retain the liquid within the lumen, and the catheter was placed in a sealed, sterile container and shipped overnight to CDC in Atlanta for processing within 24 hours of collection. At CDC, the CVC was placed into a Class II Biological Safety Cabinet, and a 1-cm segment was removed with a sterile scalpel. This segment was cut lengthwise to expose the lumen, and the individual pieces were placed into 5% glutaraldehyde (Ted Pella, Redding, CA, USA) in 0.67 M cacodylate buffer, pH 6.2, and processed for scanning electron microscopy (*20*). Samples were examined by using a Philips XL 20 Scanning Electron Microscope (FEI Company, a subsidiary of Philips, Hillsboro, OR, USA). The remaining catheter attached to the titanium port was clamped, and the outer surface was cleaned with a 70% alcohol wipe, disinfected, and processed to recover biofilm organisms (*20*). The recovered organisms were plated on trypticase soy agar containing 5% sheep blood (blood agar; Becton, Dickinson, Sparks, MD, USA). Plates were incubated for up to 72 h at 35°C and then examined. Colonies were spread onto blood agar for subculture and identified by using standard clinical microbiologic methods (*21*). Biofilm isolates were also characterized by PFGE (methods described above).

Results

A total of 12 patients with *A. xylosoxidans* bloodstream infection were found: 9 from the retrospective case–control study and 3 from the prospective study (Table). All 12 were immunocompromised. Their ages ranged from 41 to 79 years (mean 65.8 years), and 10 (83.3%) were female. Case-patients had differing underlying diagnoses and chemotherapy regimens. Case-patients had had fevers, chills, and/or rigors within minutes to days after an infusion through their CVC. Several case-patients had multiple episodes of fever and chills during and immediately after visits to Office B when their CVC was accessed for blood collection, chemotherapy, or routine flushes. For some, these symptoms were attributed to possible side effects of chemotherapy. All case-patients had visited Office B from November 12 through December 18, 2001. Case-patient 1 was hospitalized from October 12 through November 10, 2001, and had visited Office B for daily collection to monitor neutropenia from November 13 through 19, 2001. Patients with *A. xylosoxidans* bloodstream infection were treated with antimicrobial drugs and CVC removal. Available records showed case-patients were treated with piperacillin/tazobactam; 1 case-patient who was allergic to penicillin was treated with aztreonam. One patient died from underlying malignancy (end-stage pancreatic cancer).

Matched Case–Control Study

Of the 9 case-patients identified, 7 who had clear onset date of bloodstream infection symptoms were selected for the case–control study. Case-patients were younger than controls (mean age 63.5 years [range 41–73 years] and mean age 73.2 years [range 35–89 years], respectively; p = 0.047). Case-patients were significantly more likely to have a CVC than controls. Matched case–control analysis showed that all 7 case-patients versus 4 of 47 control patients had a CVC at the time of illness onset (p<0.0001). The 2 other case-patients not included in the case–control study also had CVCs. Patients with CVCs received heparin and saline flushes before and after the CVC was used for blood collection or infusions. No records documented when each of the Office B nurses accessed the CVCs. Patients without CVCs who needed only blood collection for testing did not receive any flushes; however, those without CVCs who needed blood tests before receiving an infusion received a heparin and saline flush after a peripheral intravenous line was placed. Case-patients and controls did not have statistically significant differences in peripheral leukocyte counts, intravenous medications administered, types of chemotherapy received, or underlying diseases.

Prospective Cohort Study

In February 2002, 29 patients with CVCs had blood collected for culture. Of the 3 (10%) who had positive cul-

Table. Characteristics of 12 case-patients with bacteremia caused by *Alcaligenes xylosoxidans**

Case-patient no.	Age, y	Sex	Underlying disease	Date of CVC flush	Date of onset, symptoms†	Date of blood collection‡
Case–control study						
1	70	F	Acute myelogenous leukemia	2001 Nov 19	2001 Nov 19, rigors after flush	2001 Nov 19
2	65	F	Breast cancer	2001 Dec 5	2001 Dec 5, no chart record of symptoms after flush, blood culture obtained next day	2001 Dec 6
3	73	F	Colon cancer	2001 Dec 7	2001 Dec 7, fever after flush	2001 Dec 7
4	41	F	Sickle cell disease	2001 Dec 4	2001 Dec 11, myalgias, emesis	2001 Dec 11
5	73	F	Gastric cancer	Unknown	Unknown	2001 Dec 13
6	71	M	Colon cancer	2001 Dec18	2001 Dec 19, fever, chills	2001 Dec 20
7	79	F	Pancreatic cancer	Unknown	Unknown	2001 Dec 30
8	45	F	Breast cancer	2002 Jan 10	2002 Jan 10, no chart record of symptoms after flush, blood culture obtained next day	2001 Jan 10
9	70	F	Non-Hodgkin lymphoma	2002 Jan 10	2002 Jan 10, hypotension after infusion	2002 Jan 11
Prospective study						
10	77	F	Squamous cell cancer of palate	NA	2001 Nov/Dec, intermittent nausea and weakness	2002 Jan 29
11	77	M	Gastric cancer	NA	2001 Nov/Dec, intermittent fever, chills after flushes, not reported to Office B staff	2002 Feb 7
12	49	F	Non-Hodgkin lymphoma	NA	Asymptomatic	2002 Feb 5

*CVC, central venous catheter; NA, not applicable.
†Signs and symptoms of bloodstream infection.
‡Collection of blood that had subsequent positive culture result for *A. xylosoxidans*.

ture results for *A. xylosoxidans,* chart review showed that 2 had been intermittently symptomatic (Table).

Environmental Investigation

Cultures from available open solutions in the oncology office, collected 6 weeks after the initial cluster of *A. xylosoxidans*–positive blood cultures, and environmental cultures did not grow *A. xylosoxidans*. A sample from a sterile saline bottle that was open in the infusion room was positive for *Bacillus circulans*, and a tap water sample was positive for *Moraxella* spp.

Infection Control Practices and Procedures

Office B had 10 patient examination rooms and a separate, large, open infusion room where several patients could receive chemotherapy. The infusion room contained a hood and sink for preparation of intravenous medication. Of the 4 staff members at Office B who regularly accessed CVCs; inserted peripheral intravenous catheters; collected blood; and prepared or administered chemotherapy, flushes, or intravenous medications, only 1 was a registered nurse who had a California state license. The 3 nonlicensed staff members were reported to have received nursing training in their native country but did not have documented formal training or education. One nurse wore artificial fingernails but had removed them before hand culture samples were collected; thus, the fingernails were unavailable for culture. The following breaches in infection control were noted: intravenous catheters were inserted by persons not wearing gloves; unlabeled, prefilled syringes were stored in the hood; no documentation of hood cleaning was found; open, multidose heparin vials and saline bottles, some undated, were found throughout the facility; nonhygienic material was stored in the chemotherapy medication preparation hood; and failure to wash hands between patients was noted. No pharmacists were employed at Office B. No documentation of staff training and evaluation for chemotherapy preparation or infection control competency was available.

CVC Biofilm Studies

Scanning electron microscopy of the CVC showed a biofilm that contained rod-shaped bacteria in association with fibrinlike material on the catheter surface (Figure 1). A pure bacterial culture recovered from the CVC lumen was identified as *A. xylosoxidans*.

Molecular Studies

A. xylosoxidans blood culture isolates from case-patients were indistinguishable by PFGE analysis (Figure 2); in contrast, 3 *A. xylosoxidans* isolates from a local reference laboratory had different PFGE patterns. The isolate from the CVC biofilm matched the *A. xylosoxidans* bloodstream infection outbreak strain.

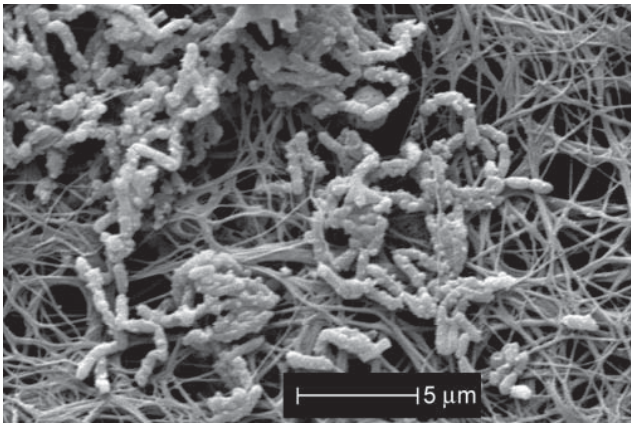


Figure 1. Scanning electron micrograph of lumen of segment of central venous catheter removed from an asymptomatic patient. Biofilm contains rod-shaped bacteria (*Alcaligenes xylosoxidans)* in association with fibrinlike material on the catheter surface.

Discussion

This large outbreak (N = 12) of *A. xylosoxidans* bloodstream infections was caused by 1 strain, which was also isolated from CVC biofilm. Symptoms of bloodstream infection probably occurred when flushes detached bacteria from the CVC biofilm. The prospective study found that 3 (10%) of 29 patients had *A. xylosoxidans*–positive blood cultures. Our case–control and prospective studies support the association of *A. xylosoxidans* bloodstream infection and CVCs, and our molecular biologic studies confirm A. *xylosoxidans* biofilm of the same outbreak strain on a CVC. *A. xylosoxidans* outbreaks reported to date have been associated with healthcare

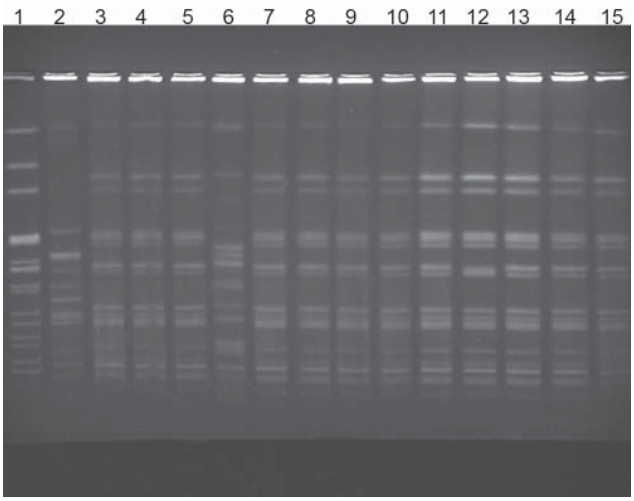


Figure 2. Pulsed-field gel electrophoresis of isolates from patients with *Alcaligenes xylosoxidans* bloodstream infection. Lane 1, laboratory standard; lanes 2 and 6, community strains of *A. xylosoxidans*; lanes 3–5 and 7–13, outbreak strains; lane 14, central venous catheter (CVC) port biofilm outbreak strain; lane 15, CVC port outbreak stain.

and contamination of hospital products (*1*,*2*,*5*,*12*–*14*), but none occurred in an outpatient setting.

The cause of this outbreak most likely was the use of contaminated multidose vials of heparin or saline flushes, leading to the formation of an *A. xylosoxidans* biofilm on CVCs. Case-patient 1 had been hospitalized from late October through early November at Hospital A. During November 13–19, 2001, this case-patient had had blood collected and her CVC line flushed numerous times at Office B; on November 19, culture result indicated *A. xylosoxidans* infection, which was successfully treated. We observed multiple breaches in infection control at Office B: use of unlabeled, prefilled syringes, poor hand hygiene, and lack of glove use. The CVC of case-patient 1 may have been flushed by using the same syringe and needle inserted into multidose vials, causing contamination of the vials. Another possible route of contamination is through artificial fingernails. A cluster of *Serratia marcescens* and *A. xylosoxidans* bacteremia cases linked to multidose heparin vials contaminated by a nurse with artificial fingernails has been reported (*22*); however, the artificial fingernails from the nurse at Office B were unavailable for testing. We suspect that multidose vials were contaminated with *A. xylosoxidans* and subsequently used on other patients from November 12 through December 18, 2001, when all case-patients had overlapping visits at Office B and received CVC flushes. A culture from an open, supposedly sterile saline bottle grew *B. circulans*, which suggests possible breaches in infection control. Multidose heparin and saline vials have been reported as the cause of outbreaks of hepatitis C (*23*,*24*), *S. marcescens* (*25*), and *Pseudomonas aeruginosa* (*26*) infections.

Although the heparin and the saline vials could have been contaminated from case-patient 1 in November, case-patients who subsequently received flushes from these vials may not have become immediately ill with symptoms of bloodstream infection. *A. xylosoxidans* biofilm could have developed on their CVCs and intermittently caused clinical illness when the CVCs were manipulated; i.e., flushing dislodged the biofilm and caused symptomatic bacteremia. Although indwelling catheters are frequently colonized with biofilm shortly after insertion (*27*), colonization does not necessarily lead to infection; bloodstream infection symptoms from an organism in contaminated intravenous solutions have been delayed for as long as 421 days after exposure (*28*). The finding of an asymptomatic patient with a CVC with *A. xylosoxidans* biofilm supports this. A number of variables may be associated with detachment of microbial cells from a biofilm (*29*), resulting in erosion or sloughing. Flushing, which could mechanically shear the biofilm, could result in detachment of cells or aggregates that could in turn colonize the bloodstream and cause signs and symptoms of bacteremia. This phenomenon has been recently reported (*28*).

The case-patients in this outbreak had their CVCs removed and were treated with antimicrobial agents. The presence of *A. xylosoxidans* biofilm and the mechanism of bloodstream infection after disruption by catheter flushing suggests that eradication of infection would require catheter removal, as reported by others (*4*,*9*). Previously, recurrent *A. xylosoxidans* bacteremia has been reported in those patients whose indwelling catheters were not removed (*11*). Formation of *A. xylosoxidans* biofilm provides an explanation for the organism most commonly causing bacteremia in patients with CVCs (*7*,*10*).

The California Code of Regulations, Title 17, Section 2500 (*30*), requires all healthcare professionals to immediately report outbreaks of any cause; however, this outbreak was not recognized early on. The initial cluster of patients at Office B had symptoms and positive blood cultures growing this uncommon organism for 6 weeks before the cases were reported to the Los Angeles County Department of Public Health. Because outpatient settings may lack surveillance systems, outbreaks may not be recognized immediately, thus potentially exposing more patients. In addition, some of the symptoms of bloodstream infection were initially attributed to side effects of chemotherapy. Because 10% of patients in our prospective cohort study had blood cultures positive for *A. xylosoxidans*, further studies are needed to determine whether active surveillance for patients with CVCs would help recognize infections.

Because we noted not only infection control breaches but also that unlicensed office personnel were manipulating the CVCs, line flushes, infusions, and blood collection through CVCs, we reported the situation to the California Medical Board and the California Department of Health Services. Although no California state regulations for infection control in outpatient physician's offices exist, the California Department of Health Services and Los Angeles County Department of Public Health recommended that the oncology office improve infection control standards; handling, storage, exposure, and access to pharmaceuticals; and improve medical record documentation. New infection control policies were established, and the office subsequently hired new, properly licensed registered nurses and nurse practitioners to handle insertion of intravenous catheters and administration of intravenous medications and chemotherapy. After proper education of the oncology office staff and removal of multidose vials of heparin and saline, no more *A. xylosoxidans* bloodstream infections were reported from Office B.

Our investigation has limitations. We did not culture *A. xylosoxidans* from the multidose vials. The original vials, used when the outbreak began, had already been discarded and were not available for testing by the time we were notified in January 2002. Our investigation was also limited by the absence of medical records indicating when

nursing staff accessed the CVCs. Although the contamination of multidose vials remains suggestive, we suspect that they were the most likely source. The outbreak ended after discontinuing their use and enacting improved infection control practices. The organism could have been introduced into multidose vials by a needle or syringe used on an infected patient or by the artificial fingernails of the nurse, through gaps in infection control.

For patients who received infusion therapy at home, receipt of therapy in an outpatient clinic or physician's office was an independent risk factor for bloodstream infection (*31*). Therefore, clinicians need to be vigilant because minor breaches in infection control can lead to large outbreaks with uncommon human pathogens, especially in patients with CVCs. Clinicians also need to ensure that appropriate infection control practices are adhered to consistently, especially in outpatient care settings, where oversight of infection control procedures may be absent. Unlike standards that exist for nursing homes or hospitals, no written standards regarding infection control in outpatient settings exist from the California Department of Health Services or the California Medical Board. However, routine monitoring and adherence to infection control could prevent outbreaks. Clinicians providing care in outpatient settings should review appropriate infection control standards and consider establishing written policies to ensure that standards are met. As healthcare delivery continues to move toward outpatient care (*32*), the lack of formal infection control procedures and accountability in the outpatient office setting can lead to large disease outbreaks (*33*,*34*); the need for oversight in this setting should be considered.

Our investigation helps characterize the mechanisms of *A. xylosoxidans* bloodstream infection in immunocompromised patients with CVCs. It provides a better understanding of how biofilm formation in a CVC can result in a clinical infectious disease process with this uncommon organism. Substantial illness and death can occur in outpatient settings that lack formal oversight. This outbreak highlights an unaddressed infection control problem in the outpatient setting for regulating agencies to further review.

Acknowledgments

We thank Ricardo Murga, Janice Carr, Bette Jensen, and Phillip K. Ng for their contributions during this outbreak investigation.

Dr Kim is an infectious disease physician at the Acute Communicable Disease Control Program, Department of Public Health, in Los Angeles County. Her research interests focus on the public health effects of infectious pathogens.

References

1. Reverdy ME, Freney J, Fleurette J, Coulet M, Surgot M, Marmet D, et al. Nosocomial colonization and infection by *Achromobacter xylosoxidans.* J Clin Microbiol. 1984;19:140–3.
2. Gahrn-Hansen B, Alstrup P, Dessau R, Fuursted K, Knudsen A, Olsen H, et al. Outbreak of infection with *Achromobacter xylosoxidans* from contaminated intravascular pressure transducers. J Hosp Infect. 1988;12:1–6. DOI: 10.1016/0195-6701(88)90115-6
3. Duggan JM, Goldstein SJ, Chenoweth CE, Kauffman CA, Bradley SF. *Achromobacter xylosoxidans* bacteremia: report of four cases and review of the literature. Clin Infect Dis. 1996;23:569–76.
4. Knippschild M, Schmid EN, Uppenkamp M, König E, Meusers P, Brittinger G, et al. Infection by *Alcaligenes xylosoxidans* subsp. *xylosoxidans* in neutropenic patients. Oncology. 1996;53:258–62.
5. Vu-Thien H, Darbord JC, Moissenet D, Dulot C, Dufourcq JB, Marsol P, et al. Investigation of an outbreak of wound infections due to *Alcaligenes xylosoxidans* transmitted by chlorhexidine in a burns unit. Eur J Clin Microbiol Infect Dis. 1998;17:724–6. DOI: 10.1007/s100960050168
6. Gómez-Cerezo J, Suárez I, Ríos JJ, Peña P, García de Miguel MJ, de José M, et al. *Achromobacter xylosoxidans* bacteremia: a 10-year analysis of 54 cases. Eur J Clin Microbiol Infect Dis. 2003;22:360–3. DOI: 10.1007/s10096-003-0925-3
7. Tsay RW, Lin LC, Chiou CS, Liao JC, Chen CH, Liu CE, et al. *Alcaligenes xylosoxidans* bacteremia: clinical features and microbiological characteristic of isolates. J Microbiol Immunol Infect. 2005;38:194–9.
8. Legrand C, Anaissie E. Bacteremia due to *Achromobacter xylosoxidans* in patients with cancer. Clin Infect Dis. 1992;14:479–84.
9. Hernandez JA, Martino R, Pericas R, Sureda A, Brunet S, Domingo-Albos A. *Achromobacter xylosoxidans* bacteremia in patient with hematologic malignancies. Haematologica. 1998;83:284–5.
10. Aisenberg G, Rolston KV, Safdar A. Bacteremia caused by *Achromobacter* and *Alcaligenes* species in 46 patients with cancer (1989–2003). Cancer. 2004;101:2134–40. DOI: 10.1002/cncr.20604
11. Shie SS, Huang CT, Leu HS. Characteristics of *Achromobacter xylosoxidans* bacteremia in northern Taiwan. J Microbiol Immunol Infect. 2005;38:277–82.
12. Reina J, Antich M, Siquier B, Alomar P. Nosocomial outbreak of *Achromobacter xylosoxidans* associated with a diagnostic contrast solution. J Clin Pathol. 1988;41:920–1. DOI: 10.1136/jcp.41.8.920-c
13. Lehours P, Rogues AM, Occhialini A, Boulestreau H, Gachie JP, Megraud F. Investigation of an outbreak due to *Alcaligenes xylosoxidans* subspecies *xylosoxidans* by random amplified polymorphic DNA analysis. Eur J Clin Microbiol Infect Dis. 2002;21:108–13. DOI: 10.1007/s10096-001-0669-x
14. Tena D, Carranza R, Barberá JR, Valdezate S, Garrancho JM, Arranz M, et al. Outbreak of long-term intravascular catheter-related bacteremia due to *Achromobacter xylosoxidans* subspecies *xylosoxidans* in a hemodialysis unit. Eur J Clin Microbiol Infect Dis. 2005;24:727–32. DOI: 10.1007/s10096-005-0028-4
15. McGuckin MB, Thorpe RJ, Koch KM, Alavi A, Staum M, Abrutyn E. An outbreak of *Achromobacter xylosoxidans* related to diagnostic tracer procedures. Am J Epidemiol. 1982;115:785–93.
16. Granowitz EV, Keenholtz SL. A pseudoepidemic of *Alcaligenes xylosoxidans* attributable to contaminated saline. Am J Infect Control. 1998;26:146–8. DOI: 10.1016/S0196-6553(98)80035-7
17. Kim MJ, Bancroft E, Mascola L, Lehnkering E, Lawani L. Outbreak of *Alcaligenes xylosoxidans* bloodstream infections in an outpatient oncology office, Los Angeles, 2002 [abstract 415]. In: Program and abstracts of the 40th annual meeting of the Infectious Diseases Society of America (Chicago). Alexandria (VA): Infectious Disease Society of America; 2002.

18. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella,* and *Shigella* for PulseNet. Foodborne Pathog Dis. 2006;3:59–67. DOI: 10.1089/fpd.2006.3.59
19. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233–9.
20. Donlan RM, Murga R, Bell M, Toscano CM, Carr JH, Novicki TJ, et al. Protocol for detection of biofilms on needleless connectors attached to central venous catheters. J Clin Microbiol. 2001;39:750–3. DOI: 10.1128/JCM.39.2.750-753.2001
21. Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, eds. Manual of clinical microbiology. 7th ed. Washington: American Society for Microbiology; 1999.
22. Gordin FM, Schultz ME, Huber R, Zubairi S, Stock F, Kariyil J. A cluster of hemodialysis-related bacteremia linked to artificial fingernails. Infect Control Hosp Epidemiol. 2007;28:743–4. DOI: 10.1086/517977
23. Krause G, Trepka MJ, Whisenhunt RS, Katz D, Nainan O, Wiersma ST, et al. Nosocomial transmission of hepatitis C virus associated with the use of multidose saline vials. Infect Control Hosp Epidemiol. 2003;24:122–7. DOI: 10.1086/502176
24. Bruguera M, Saiz JC, Franco S, Giménez-Barcons M, Sánchez-Tapias JM, Fabregas S, et al. Outbreak of nosocomial hepatitis C virus infection resolved by genetic analysis of HCV RNA. J Clin Microbiol. 2002;40:4363–6. DOI: 10.1128/JCM.40.11.4363-4366.2002
25. Tanaka T, Takahashi H, Kobayashi JM, Ohyama T, Okabe N. A nosocomial outbreak of febrile bloodstream infection caused by heparinized-saline contaminated with *Serratia marcescens*, Tokyo, 2002. Jpn J Infect Dis. 2004;57:189–92.
26. Prospero E, Barbadoro P, Savini S, Manso E, Annino I, D'Errico MM. Cluster of *Pseudomonas aeruginosa* catheter-related bloodstream infections traced to contaminated multidose heparinized saline solutions in a medical ward. Int J Hyg Environ Health. 2006;209:553–6. DOI: 10.1016/j.ijheh.2006.05.002
27. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15:167–93. DOI: 10.1128/CMR.15.2.167-193.2002
28. Centers for Disease Control and Prevention. Update: delayed onset *Pseudomonas fluorescens* bloodstream infections after exposure to contaminated heparin flush—Michigan and South Dakota, 2005–2006. MMWR Morb Mortal Wkly Rep. 2006;55:961–3.
29. Donlan RM. Biofilms: microbial life on surfaces. Emerg Infect Dis. 2002;8:881–90.
30. California Code of Regulations. Title 17, Section 2500 [cited 2007 Jun 22]. Available from http://ccr.oal.ca.gov/linkedslice/default.asp?SP=CCR-1000&Action=Welcome
31. Tokars JI, Cookson ST, McArthur MA, Boyer CL, McGeer AJ, Jarvis WR. Prospective evaluation of risk factors for bloodstream infection in patients receiving home infusion therapy. Ann Intern Med. 1999;131:340–7.
32. Jarvis WR. Infection control and changing health-care deliver systems. Emerg Infect Dis. 2001;7:170–3.
33. Samandari T, Malakamadze N, Balter S, Perz JF, Khristova M, Swetnam L, et al. A large outbreak of hepatitis B virus infections associated with frequent injections at a physician's office. Infect Control Hosp Epidemiol. 2005;26:745–50. DOI: 10.1086/502612
34. Watson JT, Jones RC, Siston AM, Fernandez JR, Martin K, Beck E, et al. Outbreak of catheter-associated *Klebsiella oxytoca* and *Enterobacter cloacae* bloodstream infections in an oncology chemotherapy center. Arch Intern Med. 2005;165:2639–43. DOI: 10.1001/archinte.165.22.2639

Address for correspondence: Moon J. Kim, Acute Communicable Disease Control Program, Los Angeles County Department of Public Health, 313 N Figueroa St, Rm 222, Los Angeles, CA 90012, USA; email: mokim@ph.lacounty.gov

Testing for Coccidioidomycosis among Patients with Community-Acquired Pneumonia

Douglas C. Chang,* Shoana Anderson,† Kathleen Wannemuehler,* David M. Engelthaler,† Laura Erhart,† Rebecca H. Sunenshine,† Lauren A. Burwell,* and Benjamin J. Park*

Coccidioidomycosis is a common cause of community-acquired pneumonia (CAP) in disease-endemic areas. Because testing rates influence interpretation of reportable-disease data and quality of CAP patient care, we determined the proportion of CAP patients who were tested for *Coccidioides* spp., identified testing predictors, and determined the proportion of tested patients who had positive coccidioidomycosis results. Cohort studies to determine the proportion of ambulatory CAP patients who were tested in 2 healthcare systems in metropolitan Phoenix found testing rates of 2% and 13%. A case-control study identified significant predictors of testing to be age ≥18 years, rash, chest pain, and symptoms for ≥14 days. Serologic testing confirmed coccidioidomycosis in 9 (15%) of 60 tested patients, suggesting that the proportion of CAP caused by coccidioidomycosis was substantial. However, because *Coccidioides* spp. testing among CAP patients was infrequent, reportable-disease data, which rely on positive diagnostic test results, greatly underestimate the true disease prevalence.

Coccidioidomycosis (valley fever) is a disease caused by *Coccidioides* spp., dimorphic fungi that thrive in the alkaline soil of warm, arid climates (*1*). Infection may occur when conidia in disrupted soil are inhaled. Coccidioidomycosis-endemic areas include the southwestern United States, parts of Mexico, and Central and South America. In the United States, these areas include parts of Arizona, California, New Mexico, Nevada, Texas, and Utah (*1*).

The clinical manifestations of coccidioidomycosis have been well established (*2*–*4*); 1–3 weeks after a person inhales the spores, most persons with symptomatic infection will have a clinical syndrome consistent with community-acquired pneumonia (CAP) (*3*). Serologic testing is the most frequently used method for diagnosis of primary pulmonary coccidioidomycosis (*3*,*5*). For most patients, serologic reactivity ends after a few months unless infection is active.

Although 95% of symptomatic pulmonary infections are self-limiting and resolve after several weeks or months without antifungal therapy, ≈5% progress to asymptomatic residua, such as pulmonary nodules or cavities (*2*). Among all recognized infections, extrapulmonary disease involving the meninges, bones and joints, skin, and soft tissues occurs in <5% (*2*). Risk factors for severe or disseminated infection include immunosuppression, diabetes, preexisting cardiopulmonary disease, second- or third-trimester pregnancy, and African or Filipino descent (*6*).

Previous studies suggest that the true prevalence of coccicioidomycosis is substantially underestimated (*6*,*7*). One study, which used rates of skin-test conversion, estimated that clinical illness would develop in ≈30,000 persons per year in southern Arizona (*7*). Another study found that coccidioidomycosis was serologically confirmed for 29% of CAP patients in primary care clinics in Tucson, Arizona (*8*). To understand more about the unmeasured prevalence of disease, we evaluated *Coccidioides* spp.testing practices for ambulatory clinic patients with CAP in Maricopa County, which encompasses most of metropolitan Phoenix in Arizona, a state where coccidioidomycosis is reportable. Our objectives were to estimate the proportion of patients with CAP who were tested for coccidioidomycosis, to determine predictors of testing, and to determine the proportion of CAP patients who had coccidioidomycosis. To accomplish our objectives, we performed 3 related studies: a data analysis, a retrospective cohort study, and a case–control study.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and †Arizona Department of Health Services, Phoenix, Arizona, USA

DOI: 10.3201/eid1407.070832

Methods

Descriptive Epidemiology

To describe the epidemiology of coccidioidomycosis in metropolitan Phoenix, we analyzed data from the National Electronic Telecommunication System for Surveillance. We calculated county-specific and age group–specific incidence rates for 1999–2004. Population denominators were obtained from the US Census Bureau (http://quickfacts.census.gov/qfd/index.html).

Retrospective Cohort Studies

To determine the proportions of CAP patients tested for *Coccidioides* spp., we performed retrospective cohort studies in 2 healthcare systems (systems A and B) in metropolitan Phoenix. System A served large numbers of patients without private insurance, whereas system B was associated with an insurance company. System A comprised 13 clinics and system B comprised 17 (Table 1). Race and ethnicity data were available for patients in system A (19% white, 6% black, 69% Hispanic, 4% other) but not system B.

Administrative databases from both systems were screened to identify outpatient visits from January 1, 2003, through December 31, 2004, in which patients were assigned primary or secondary codes from the International Classification of Diseases, 9th revision (ICD-9), beginning with 486 (pneumonia, organism unspecified). Patient charts were selected by simple random sampling for chart review to determine whether patients met inclusion criteria. Patients were included if their initial visit was as an outpatient, they had no history of coccidioidomycosis, and they had CAP as defined by the clinician. Patients were excluded if they were hospitalized or had been residents of a long-term care facility within 14 days of symptom onset. Demographic, clinical, diagnostic data (including *Coccidioides* spp. testing), and outcomes were extracted from medical records for all subsequent visits within 2 months after the initial clinic visit.

Preliminary results, obtained by using Current Procedural Terminology codes for *Coccidioides* spp. serologic testing (86635, 86329, 86331, or 86171), indicated that 13% of patients in system B were being tested for *Coccidioides* spp. compared with 1% in system A. Assuming $\alpha = 0.05$ and a power of 80%, a sample size of 86 was needed for each cohort to show a difference in testing frequency between the 2 systems.

Case–Control Study

To determine factors associated with coccidioidomycosis testing (testing predictors) among CAP patients, we conducted a case–control study in system B. We identified all ambulatory patients who had visited system B in 2003 and 2004 and had an ICD-9 code for "pneumonia, organism unspecified" and a Current Procedural Terminology code for *Coccidioides* spp. serologic testing. Patients with CAP were defined by the same criteria used in the cohort studies. Case-patients were defined as CAP patients who had received *Coccidioides* spp. serologic testing, regardless of test result. Controls were defined as patients who met the definition for CAP but had not received *Coccidioides* spp. serologic testing. A simple random sample of visits was reviewed to confirm inclusion criteria and to confirm *Coccidioides* spp. serologic testing associated with the pneumonia.

Statistical Analysis

All data were entered into Epi Info (Centers for Disease Control and Prevention [CDC], Atlanta, GA, USA). Analysis was performed in Epi Info, SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA), and StatXact 6 (Cytel Software Corp., Cambridge, MA, USA). Confidence intervals (CIs) on difference in proportions were determined by assuming 2 independent binomial proportions and using an exact method. The Kruskal-Wallis test was used to compare continuous variables. Univariate logistic regression models related the dependent variable (a clinician ordering a test for *Coccidioides* spp.) to independent variables such as age ($\geq$18 years vs. <18 years); reported rash; chest pain; symptoms $\geq$14 days, as well as other demographic or clinical symptom characteristics. A final multivariable model was chosen by using the stepwise selection procedure. Independent variables that remained significant ($\alpha<0.05$) while other significant variables were controlled for were included in the final model. Exact 95% CIs are reported because of low observed cell counts.

Results

Descriptive Epidemiology

In 2004, Maricopa County had the most reported cases of coccidioidomycosis in Arizona (2,704 cases), followed by Pima and Pinal Counties (715 and 164 cases, respectively). Compared with other counties in Arizona,

Table 1. Characteristics of healthcare systems selected for retrospective cohort studies, Maricopa County, Arizona

Characteristic	System A	System B
Primary care	Yes	Yes
Subspecialty care	Yes	Yes
No. clinics	13	17
Associated with hospital	Yes (public)	No
Racial and ethnic minorities	Majority	Data not available
Insurance	Many without insurance	Most privately insured

Maricopa, Pima, and Pinal Counties also had the highest incidence rates (77, 77, and 75 cases per 100,000 persons, respectively). The incidence rate in Maricopa County has increased from 42 per 100,000 persons in 1999 to 77 cases per 100,000 persons in 2004. Maricopa County shows a seasonal pattern with cases peaking in the winter season (Figure), similar to findings reported elsewhere (*9*). Coccidioidomycosis was reported in every 5-year age category; the incidence rate for coccidioidomycosis was 8/100,000 for those <5 years of age and increased steadily with each age group; incidence for persons 55–64 years of age was the highest (166/100,000). Groups of persons ≥65 years of age also had high incidence rates (65–74 years, 161/100,000; 75–84 years, 147/100,000; ≥85 years, 153/100,000).

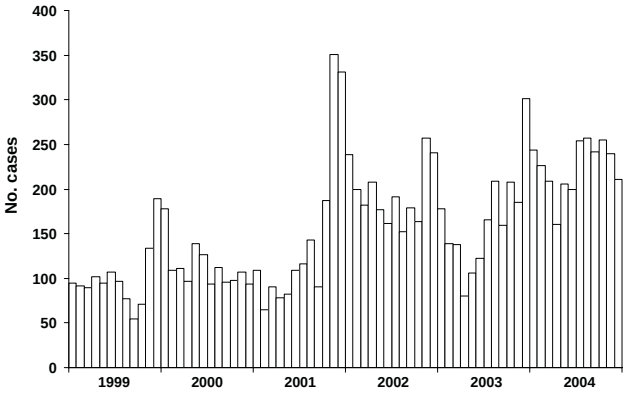


Figure. Coccidioidomycosis cases reported by month, Maricopa County, Arizona, 1999–2004.

Retrospective Cohort Studies

Participants

In system A, 619 visits for "pneumonia, organism unspecified" were identified, from which 132 (21%) were sampled for chart review. From the 132, 66 (50%) were excluded: 11 had no charts available, 21 were miscoded or did not have a clinical diagnosis of pneumonia, 30 were initially hospitalized, and 4 had been residents of long-term care facilities or hospitalized within the 14 days before symptom onset. The remaining 66 patients were confirmed by chart review to have CAP and were included.

In system B, 14,695 visits for "pneumonia, organism unspecified" were identified, from which 159 (1%) were sampled for chart review. From the 159, 72 (46%) were excluded: 6 had no charts available, 25 did not have clear documentation of pneumonia, 37 were initially hospitalized, 1 had been hospitalized within 14 days before symptom onset, and 3 had a history of coccidioidomycosis. The remaining 87 patients were therefore included.

Patient Characteristics and Clinical Description (Table 2)

Of CAP patients, 11% of those in system A were <18 years of age compared with 37% in system B (difference 26%, 95% CI 12%–39%, p<0.01). Patients in system A were significantly more likely than patients in system B to have been seen initially in an emergency department (26% vs. 6%, difference 20%, 95% CI 7%–33%, p<0.01).

Clinical signs and duration of symptoms before the initial visit were similar for patients in both systems. In systems A and B, respectively, cough was the most commonly reported symptom (82% and 79%), followed by fever (33% and 58%) and dyspnea (27% and 26%). Approximately 50% of patients in each group had focal findings on lung examination.

In terms of coexisting conditions, 38% of patients in system A had diabetes compared with 8% in system B (difference 30%, 95% CI 16%–43%, p<0.01); diabetes is a risk factor for severe pneumonia and for complicated *Coccidioides* spp. infection (*10*). Similar proportions of those with asthma or chronic obstructive pulmonary disease were found in both systems. Few patients had a diagnosis of HIV, pregnancy, or organ transplantation, and few were receiving immunosuppressive medications.

Chest radiographs were less likely to have been taken for patients in system A than for patients in system B (23% vs. 83%, difference 60%, 95% CI 47%–72%, p<0.01). Of those in both systems who did have chest radiographs taken, similar proportions had findings consistent with pneumonia (78% and 81%, respectively). All patients in both systems received treatment with antibacterial drugs.

Coccidioides spp. serologic testing was low overall for patients in systems A (1/66, 2%, 95% CI 0.04%–8%) and B (11/87, 13%, 6%–22%). Patients in system A were significantly less likely than patients in system B to have had *Coccidioides* spp. serologic testing performed at any point during the clinical course of disease (difference 11%, 95% CI 3%–20%, p = 0.01). In system B, 7 (64%) of 11 tested patients were tested during a follow-up visit rather than at the initial visit. Of those 11 patients, 1 (9%) had a reactive serologic test result for *Coccidioides* spp. Median duration of symptoms before testing for patients in system B was 27 days (range 1–99 days); most patients (64%) were tested after symptoms had been present for at least 2 weeks.

Patient outcomes were similar. In system A, 2 (3%) were known to have been hospitalized for worsening of pneumonia, and no patients were known to have died. In system B, 6 patients (7%) were hospitalized for worsening pneumonia, and 2 died; cause-of-death data were unavailable.

Case–Control Study

Of 14,695 potential CAP-patient visits in system B, we randomly selected 60 case-patients (i.e., those with CAP who had been tested for *Coccidioides* spp.) and 76 controls

Table 2. Demographic and clinical characteristics of patients with community-acquired pneumonia included in retrospective cohort studies, Maricopa County, Arizona, January 2003–December 2004*

Patient characteristic	System A (% or range), n = 66	System B (% or range), n = 87	Absolute difference in percentages (95% CI)*
Evaluated initially in emergency department	17 (26)	5 (6)	20 (7–33)†
Median age, y (range)	54 (6–90)	37 (0–86)	
Age <18 y	7 (11)	32 (37)	26 (12–39)†
Male	30 (46)	47 (54)	NS
Median no. days of symptoms before 1st visit	8 (1–30)	7 (1–60)	NS
Symptoms for ≥21 d at time of 1st visit	4 (9)	6 (7)	NS
Symptoms			
Fever	22 (33)	50 (58)	NS
Chills	5 (8)	12 (14)	NS
Night sweats	0	4 (5)	NS
Myalgias	2 (3)	3 (3)	NS
Fatigue	4 (6)	4 (5)	NS
Cough	54 (82)	69 (79)	NS
Dyspnea	18 (27)	23 (26)	NS
Chest pain	10 (15)	11 (13)	NS
Wheezing	8 (12)	9 (10)	NS
Signs			
Temperature >100.4°F	10 (15)	24 (28)	NS
Tachycardia	8 (12)	11 (13)	NS
Focal lung examination	37 (56)	44 (51)	NS
Hypoxia	0	0	NS
Rash	0	0	NS
Immunosuppressive medication	2 (3)	1 (1)	NS
Coexisting conditions			
Asthma	10 (15)	23 (26)	NS
Chronic obstructive pulmonary disease	13 (20)	12 (14)	NS
Diabetes mellitus	25 (38)	7 (8)	30 (16–43)†
HIV infection	0	1 (1)	NS
Pregnancy	1 (2)	0	NS
Transplant	1 (2)	0	NS
Malignancy	1 (2)	3 (3)	NS
Diagnostic testing, noncoccidioidal			
Chest radiograph	23 (35)	83 (95)	61 (47–72)†
Radiographically proven pneumonia	18 (27)	67 (83)	50 (35–63)†
Treatment and outcome			
Antibacterial drugs	66 (100)	87 (100)	NS
Follow-up visits			
None	17 (26)	27 (31)	NS
1	34 (52)	27 (31)	NS
2	5 (8)	13 (15)	NS
≥3	10 (15)	20 (23)	NS
Hospital admissions	2 (3)	6 (7)	NS
Died	0 (0)	2 (2)	NS
Coccidioides spp. serologic testing			
At any visit	1 (2)	11 (13)	11 (3–20)‡
During follow-up visit	0	4 (5)	NS
Reactive results	0	1 (1)	NS
Median no. days before testing	12	27 (1–99)	–
Symptoms ≥14 days before testing	0	7 (64)	NS
Diagnosis of coccidioidomycosis, any technique	0	1 (1)	NS

CI, confidence interval; NS, not significant. CIs on difference in percentages were performed by using an exact method with 2 independent binomial proportions.
†$p<0.01$.
‡$p\leq0.05$.

(i.e., those with CAP who had not been tested). According to univariate analysis, patients who had chest pain (odds ratio [OR] 4.6, 95% CI 1.8–11.8), rash (OR undefined, 95% CI 1.2–undefined), or those with symptom duration ≥14 days (OR 5.8, 95% CI 2.1–15.7) were significantly more likely to have been tested (Table 3). Additionally, patients ≥18 years of age (OR 5.5, 95% CI 2.1–15.3) and those who had diabetes or were receiving an immunosuppressive medication (OR 3.6, 95% CI 1.0–16.5) were significantly more likely to have been tested.

The multivariate model identified the following as being significantly more likely to have been tested for coccidioidomycosis: adult patients (adjusted OR 5.3, 95% CI 1.5–24.0) and those who reported rash (adjusted OR 21.1, 95% CI 2.2–∞), chest pain (adjusted OR 3.9, 95% CI 1.2–13.8), or symptoms for ≥14 days (adjusted OR 4.1, 95% CI 1.3–14.2). The Hosmer-Lemeshow goodness-of-fit test showed no evidence of a lack of fit ($p = 0.8$).

Of the 60 case-patients who were tested for *Coccidioides* spp., 9 (15%, 95% CI 8%–26%) had positive results. Of these 9, 3 had immunoglobulin (Ig) M by enzyme immunoassay alone, 3 had IgM and IgG by enzyme immunoassay (IgG titers 4, 8, and 8 by complement fixation), 1 had IgM and IgG by immunodiffusion, 1 had a single high IgG titer (128), and another had a rising IgG titer (<2 initial; 16 at 4 weeks).

Discussion

Our study directly measured serologic testing practices for coccidioidomycosis. The proportion of ambulatory patients with CAP who were tested for *Coccidioides* spp. was low in this coccidioidomycosis-endemic area.

Because incidence rates of CAP in this area of the United States are not available, the number of CAP patients who do not receive serologic testing for *Coccidioides* spp. cannot be estimated. However, if incidence rates are comparable to those in other parts of the country (*6*), the number of patients with CAP who are not tested for coccidioidomycosis would be high. If CAP is the result of coccidioidomycosis in as many as 10%–15% of these untested patients, then large numbers of patients would remain undiagnosed.

According to recently published Infectious Disease Society of America (IDSA) guidelines, the benefit of antifungal therapy for uncomplicated respiratory *Coccidioides* spp. infection is unknown (*6*). However, treatment is more likely to benefit groups at risk for severe or disseminated infection (*6*). Although these groups are especially likely to benefit from early testing for coccidioidomycosis, other benefits of early diagnosis may exist for all patients with coccidioidomycosis, regardless of risk for severe disease. Such benefits may include avoidance of unnecessary use of antibacterial agents, earlier identification of complications, decreased need for further expensive diagnostic studies, and reduction of patient anxiety (*3*).

Reasons that CAP patients may not be tested for *Coccidioides* spp. are unclear but are likely complex. First, professional consensus for optimal testing practices may have been lacking. Although guidelines developed by national professional organizations for the management of CAP were available during the study period (*11–13*), these guidelines did not directly address the best strategy for *Coccidioides* spp. serologic testing (*11–13*). IDSA guidelines for the treatment of coccidioidomycosis also did not clearly recommend serologic testing for patients with CAP (*6*,*14*). The most recent IDSA/American Thoracic Society guidelines for CAP, published after the study period, now recommend evaluating travel history or exposure to disease-endemic area during the initial assessment rather than waiting for a failed response to therapy (*15*); these guidelines may lead to increased testing for coccidioidal CAP. Second, physicians may be unaware of the benefits of early testing or the possible high prevalence of coccidioidomycosis in those with CAP in Arizona and therefore may not understand the utility of testing. Regardless of the reasons, the lack of testing in the presence of widespread disease hampers epidemiologic understanding of this disease and subsequently may affect public health decisions related to

Table 3. Characteristics of patients with community-acquired pneumonia (CAP) who were tested for coccidioidomycosis, Maricopa County, Arizona, January 2003–December 2004*

Characteristic	Case-patients, no. (%), n = 60	Controls, no. (%), n = 76	Odds ratio (95% CI)	Adjusted odds ratio† (95% CI)
Age ≥18 y	53 (88)	44 (58)	5.5 (2.1–15.3)‡	5.3 (1.5–24.0)
Male	28 (55)	40 (53)	0.9 (0.5–1.9)	NS
Chest pain	19 (32)	7 (9)	4.6 (1.8–11.8)‡	3.9 (1.2–13.8)
Rash	5 (8)	0	Undefined (1.2–Undefined)‡	21.1§ (2.2–undefined)
Diabetes mellitus or immunosuppressive condition	10 (17)	4 (5)	3.6 (1.0–16.5)‡	NS
Symptoms ≥14 d	20 (33)	6 (8)	5.8 (2.1–15.7)‡	4.1 (1.3–14.2)

*CI, confidence interval; NS, not significant. Case-patients were patients who had CAP and had received *Coccidioides* spp. serologic testing, regardless of test result; controls were patients who had CAP but had not received *Coccidioides* spp. serologic testing.
†Adjusted odds ratios and exact 95%CI from a multivariable logistic regression model.
‡Significant ($p<0.05$) according to univariate analysis.
§Median unbiased estimate of the adjusted odds ratio.

resource allocation to control disease, educate physicians, and develop a vaccine.

Our data also illustrate the marked differences in process of care for ambulatory patients with CAP in different health systems. Patients in the uninsured population, system A, were less likely to be tested than patients in the primarily insured population, system B. This disparity is evidenced by the higher proportion of CAP patients in the insured system who received chest radiography in addition to serologic testing. Public health officials may be able to address these disparities by providing general recommendations for diagnostic testing of patients with CAP in coccidioidomycosis-endemic areas; process-of-care measures such as chest radiography and *Coccidioides* spp. serologic testing may help determine effectiveness of such interventions.

Of the tested CAP patients in system B, 15% had serologic evidence of recent *Coccidioides* spp. infection; this proportion is much lower than that (29%) found in a recently reported study (*8*). Several differences may explain this discrepancy. First, our study population was located in a different area of Arizona. Second, our definition of CAP differed from the definition used in the other study and is likely more representative of actual CAP found in outpatient practices (*8*). However, our proportion may overestimate the true proportion of CAP caused by coccidioidomycosis because testing in our cohort was subject to a decision made by the treating physician. Nevertheless, the high proportion could signify that a large number of pulmonary coccidioidomycosis diagnoses are likely missed in Maricopa County alone and that the overall extent of pulmonary coccidioidomycosis is higher than that indicated by reportable disease data. Further studies are needed to better quantify the extent of disease.

Our study has several limitations. First, in contrast to definitions used in many studies, our definition of CAP included patients whose diagnosis was made by a clinician without a chest radiograph or with a negative chest radiograph. However, although some patients may not have truly had CAP, our definition reflects what clinicians actually believed they were treating, which is clinically relevant to whether a diagnostic test is ordered. Second, although the study populations were geographically dispersed throughout metropolitan Phoenix and included varied population segments, they may not be generalizable to populations in health systems in other areas of Arizona. Third, at system B, controls were inadvertently oversampled during 2003, so we were unable to include visit year in our analysis. However, it is unlikely that the biased selection based on year led to substantial bias for other variables such as age, sex, clinic location, signs, symptoms, coexisting medical conditions, or testing. Fourth, data on socioeconomic status were not available for either system, and race or ethnicity data were not available from system B. Fifth, because our study evaluated ambulatory rather than hospitalized patients with CAP, our conclusions cannot be generalized to the hospital, where testing practices are likely to differ.

Our study shows that testing for *Coccidioides* spp. among ambulatory patients with CAP is infrequent in metropolitan Phoenix. Providers in metropolitan Phoenix and other coccidioidomycosis-endemic areas should consider testing patients with CAP for coccidioidomycosis. Further epidemiologic studies are needed to better determine the true extent of pulmonary coccidioidomycosis.

Acknowledgments

We thank Andy Roher and Elizabeth Smith for their assistance with data abstraction and Kenneth Komatsu, Miriam Vega, Emma Viera Negron, Gordon Jensen, and Rick Weber for assistance with study design and implementation.

All financial support for this study was provided by CDC and the Arizona Department of Health Services.

Dr Chang is a medical epidemiologist in the Epidemic Intelligence Service, Mycotic Diseases Branch, at CDC. His research focuses on fungal diseases and infections among transplant recipients.

References

1. Pappagianis D. Epidemiology of coccidioidomycosis. Curr Top Med Mycol. 1988;2:199–238.
2. Chiller TM, Galgiani JN, Stevens DA. Coccidioidomycosis. Infect Dis Clin North Am. 2003;17:41–57, viii. DOI: 10.1016/S0891-5520(02)00040-5
3. Galgiani JN. Coccidioidomycosis: a regional disease of national importance. Rethinking approaches for control. Ann Intern Med. 1999;130:293–300.
4. Stevens DA. Coccidioidomycosis. N Engl J Med. 1995;332:1077–82. DOI: 10.1056/NEJM199504203321607
5. Pappagianis D, Zimmer BL. Serology of coccidioidomycosis. Clin Microbiol Rev. 1990;3:247–68.
6. Galgiani JN, Ampel NM, Blair JE, Catanzaro A, Johnson RH, Stevens DA, et al. Infectious Diseases Society of America. Coccidioidomycosis. Clin Infect Dis. 2005;41:1217–23. DOI: 10.1086/496991
7. Dodge RR, Lebowitz MD, Barbee R, Burrows B. Estimates of *C. immitis* infection by skin test reactivity in an endemic community. Am J Public Health. 1985;75:863–5.
8. Valdivia L, Nix D, Wright M, Lindberg E, Fagan T, Lieberman D, et al. Coccidioidomycosis as a common cause of community-acquired pneumonia. Emerg Infect Dis. 2006;12:958–62.
9. Park BJ, Sigel K, Vaz V, Komatsu K, McRill C, Phelan M, et al. An epidemic of coccidioidomycosis in Arizona associated with climatic changes, 1998–2001. J Infect Dis. 2005;191:1981–7. DOI: 10.1086/430092
10. Santelli AC, Blair JE, Roust LR. Coccidioidomycosis in patients with diabetes mellitus. Am J Med. 2006;119:964–9. DOI: 10.1016/j.amjmed.2006.03.033
11. Bartlett JG, Breiman RF, Mandell LA, File TM Jr. Community-acquired pneumonia in adults: guidelines for management. The Infectious Diseases Society of America. Clin Infect Dis. 1998;26:811–38. DOI: 10.1086/513953

12. Mandell LA, Bartlett JG, Dowell SF, File TM Jr, Musher DM, Whitney C. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. Clin Infect Dis. 2003;37:1405–33. DOI: 10.1086/380488
13. Niederman MS, Mandell LA, Anzueto A, Bass JB, Broughton WA, Campbell GD, et al. Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. Am J Respir Crit Care Med. 2001;163:1730–54.
14. Galgiani JN, Ampel NM, Catanzaro A, Johnson RH, Stevens DA, Williams PL. Practice guideline for the treatment of coccidioidomycosis. Infectious Diseases Society of America. Clin Infect Dis. 2000;30:658–61. DOI: 10.1086/313747
15. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, et al. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis. 2007;44(Suppl 2):S27–72. DOI: 10.1086/511159

Address for correspondence: Douglas C. Chang, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop C09, Atlanta, GA 30333, USA; email: douglas.chang1@ihs.gov

Determinants of Cluster Size in Large, Population-Based Molecular Epidemiology Study of Tuberculosis, Northern Malawi

Judith R. Glynn,* Amelia C. Crampin,*† Hamidou Traore,* Steve Chaguluka,† Donex T. Mwafulirwa,† Saad Alghamdi,* Bagrey M.M. Ngwira,† Malcolm D. Yates,‡ Francis D. Drobniewski,‡ and Paul E.M. Fine*

Tuberculosis patients with identical strains of *Mycobacterium tuberculosis* are described as clustered. Cluster size may depend on patient or strain characteristics. In a 7-year population-based study of tuberculosis in Karonga District, Malawi, clusters were defined by using IS*6110* restriction fragment length polymorphism, excluding patterns with <5 bands. Spoligotyping was used to compare strains with an international database. Among 682 clustered patients, cluster size ranged from 2 to 37. Male patients, young adults, and town residents were over-represented in large clusters. Cluster size was not associated with HIV status or death from tuberculosis. Spoligotypes from 9 (90%) of 10 large cluster strains were identical or very similar (1 spacer different) to common spoligotypes found elsewhere, compared with 37 (66%) of 56 of those from nonclustered patients (p = 0.3). Large clusters were associated with factors likely to be related to social mixing, but spoligotypes of common strains in this setting were also common types elsewhere, consistent with strain differences in transmissibility.

Molecular techniques, in particular restriction fragment length polymorphism (RFLP) based on the IS*6110* insertion element, are used to define clusters of isolates of *Mycobacterium tuberculosis* with identical DNA fingerprints. Many studies have investigated risk factors for clustering, but relatively little is known about the determinants of cluster size (*1*,*2*). The size of clusters could depend on factors favoring transmission or on differences in the strains themselves. *M. tuberculosis* strains found in persons with smear-positive disease, many contacts, or delays in diagnosis and effective treatment are particularly likely to be transmitted. Some strains may be inherently more transmissible than others, perhaps because they are particularly likely to give rise to sputum smear–positive disease, they are associated with a more insidious onset of clinical symptoms (so patients are infectious for longer), or they are more virulent and are therefore more likely to give rise to secondary cases within the period studied (*3*). Large clusters may also be observed if the strain has a particularly stable RFLP pattern; this may be more likely for strains with few bands.

Epidemiologic differences can be explored by examining risk factors for cluster size. Giordano et al. (*1*) hypothesized that cluster size would be related to duration of symptoms. Those researchers found no evidence of this but did find inverse associations with age and HIV status in a population-based study in Texas in the United States. Strain-related differences are likely if the same strains give rise to large clusters in unrelated populations. The ubiquity of the Beijing family of strains has led to speculation that they may be particularly virulent or transmissible (*4*).

In a population-based study of the molecular epidemiology of tuberculosis in northern Malawi, we found that clustering was associated with young age, female sex, area of residence, and, in older adults, HIV positivity (*5*). We explored the determinants of cluster size and the characteristics of the larger clusters.

*London School of Hygiene and Tropical Medicine, London, UK; †Karonga Prevention Study, Chilumba, Malawi; and ‡Health Protection Agency, London

DOI: 10.3201/eid1407.060468

Methods

As part of the Karonga Prevention Study, northern Malawi, all persons with suspected tuberculosis at peripheral clinics and the district hospital are seen by project staff. Sputum is collected for smear and culture; lymph node and pleural and peritoneal aspirates are also cultured, when available. Cultures are set up in the project laboratory in Malawi, and those macroscopically consistent with *M. tuberculosis* are sent to the Health Protection Agency Mycobacterium Reference Unit, London, United Kingdom, for species identification and drug resistance testing. HIV testing is conducted after counseling, if consent is given. Patients are treated for tuberculosis according to Malawi government guidelines (*6*).

DNA fingerprinting using IS*6110* RFLP has been conducted on isolates from patients who have been diagnosed since late 1995, following standard procedures (*7*). Patients whose disease was diagnosed up to early 2003 were included in this analysis. RFLP patterns were compared by using computer-assisted (Gelcompar 4.1; Applied Maths, Kortrijk, Belgium) visual comparison. Laboratory error was thought likely if isolates with identical RFLP patterns were isolated on the same day from patients with no known epidemiologic relationship if, in addition, there was no other laboratory evidence of tuberculosis, or if they were the only 2 examples of this RFLP pattern, or if the patients had other isolates with different patterns (*8*). After likely laboratory errors were excluded, RFLP patterns shared by >1 patient were classified as clustered. Some patients had >1 isolate. To define whether a strain was clustered and to determine the size of the cluster, patients were included more than once if they had >1 RFLP pattern. Thereafter, patients were only included once, for their first episode of tuberculosis for which an RFLP result was available.

Spoligotyping (*9*) was performed on at least 2 isolates of clusters containing at least 15 patients, to enable comparison of strains with international databases (*10*,*11*). Changes in the proportion of tuberculosis cases caused by each of these large cluster strains over time was examined, by using the Fisher exact test to compare proportions and the χ^2 test for linear trend. Spoligotyping was also performed on unique (not clustered) strains from patients with smear-positive tuberculosis in 1998 or 1999, as examples of strains that had apparently not spread in the population; and from all positive cultures from 2002. Previously identified spoligotypes were defined as widespread if the international database described them as both "ubiquitous" and "recurrent," "common," or "epidemic."

Analysis of cluster size excluded unique strains and strains with <5 bands on the RFLP (because patterns with few bands are insufficiently discriminatory). Cluster size was divided into 4 groups (Table 1), and associations with cluster size were determined by using maximum-likelihood ordered logistic regression with the ologit command in STATA (*12*). With this method, the odds ratios calculated represent the summary relative odds of larger clusters compared to smaller clusters across the 4 groups. This method was used in preference to linear regression because cluster size is not normally distributed, and in preference to logistic regression because it avoids arbitrary dichotomization of cluster size. All available risk factors for cluster size were assessed individually (Table 1), and factors that were significant at the 5% level, after adjusting for other factors, or that confounded other variables were retained in the final model. The molecular epidemiologic work of the Karonga Prevention Study was approved by the Malawi National Health Sciences Research Committee and the ethics committee of the London School of Hygiene and Tropical Medicine.

Results

Over the study period, 1,248 cases of culture-positive tuberculosis were diagnosed in patients in Karonga District. RFLP results were available on 1,194 isolates from 1,044 patients. After we excluded 25 isolates because laboratory error was suspected (*8*), there were results for 1,029 patients. Eighty-one had <5 bands so they were excluded. Of the remaining 948 patients, 682 (72%) were clustered and form the basis of this analysis.

Cluster size varied from 2 to 37. The determinants of cluster size are shown in Table 1. Older patients were less likely than younger patients to be in large clusters. Male patients were more likely than female patients to be in large clusters, and there was variation by geographic area. Cluster size was not statistically associated with HIV status, type of tuberculosis, previous tuberculosis, or drug resistance. Patients in small clusters were as likely to die during treatment as those in large clusters. In the multivariate analysis, the results were similar (Table 2), with significant associations with age, sex, and area of residence. The results were unchanged by adjusting for year or for RFLP band number. None of the other factors shown in Table 1 was associated with clustering after we adjusted for possible confounders. Repeating the analysis with different categorizations of cluster size gave similar results (not shown).

All of the large cluster strains (≥15 people) were found in at least 4 of the 6 geographic areas of the district, and most were found throughout the district. The distributions of the 4 largest clusters are shown in the Figure. Patients with strains from most of the large clusters were present in the district throughout the study period. Trends over time for strains involving at least 15 people are shown in Table 3. Only 1 strain, kps121, showed statistically significant changes over time; it appeared to be decreasing.

Spoligotypes from large clusters (≥15 people) were compared with the international database (*10*,*11*). The re-

sults, displayed according to the octal code, are shown in Table 4 (*13*). Six of the large cluster strains had patterns identical or very similar to spoligotype 59, which is classified as ubiquitous and recurrent (*10*,*11*). These 6 RFLP-defined strains (kps10, 12, 20, 21, 41, and 64) had similar RFLP patterns, with a similarity coefficient of 79% (with 1% position tolerance).

The spoligotypes for RFLP-defined strains kps104, kps44, and kps97 were also identical or similar to previously described widespread spoligotypes, types 21, 53, and 1 (Beijing), respectively. The spoligotype for strain kps121, spoligotype129, was not similar to any widespread types.

The spoligotypes from the RFLP-defined large cluster strains were compared with spoligotypes from patients with positive cultures in 2002, and from patients with smear-positive tuberculosis and unique RFLP patterns in 1998 through 1999. Overall, 9 (90%) of 10 of the large cluster strains had spoligotypes that were identical to, or only 1 spacer different from, previously described widespread spoligotypes. For the patients from 2002, this proportion was 90 (71%) of 126 ($p = 0.3$ when compared to the large cluster strains), and for the smear-positive unique strains, it was 37 (66%) of 56 ($p = 0.3$ compared to the large cluster strains).

All the spoligotypes that were found in the RFLP-defined large cluster strains were also found among (RFLP-defined) unique strains. Seventeen of the unique strains had spoligotype 59, and 2 others had closely related patterns (i.e., 1 spacer different); 1 had spoligotype 21, and 1 had a closely related pattern; 4 had spoligotype 53, and 2 had closely related patterns; and 6 had spoligotype 129. Of the 56 patients from 1998 to 1999, none had Beijing spoligotypes, but we have previously described strains with Beijing spoligotypes and unique RFLP patterns in this population (*14*).

The spoligotypes found in the large cluster strains were also common among the unselected patients from

Table 1. Associations between patient characteristics and cluster size, *Mycobacterium tuberculosis*, bivariate analysis, Malawi

		Cluster size, %					
Characteristic	N	2–4 (n = 186)	5–10 (n = 196)	11–20 (n = 173)	>20 (n = 127)	Odds ratio (95% CI)*	p value
Age, y							0.05
<25	111	27.9	25.2	25.2	21.6	1	
25–34	266	24.4	26.3	27.8	21.4	1.1 (0.74–1.7)	
35–44	160	26.3	32.5	25.0	16.3	0.87 (0.56–1.3)	
≥45	145	33.1	31.7	21.4	13.8	0.66 (0.42–1.0)	
Sex							0.1
F	386	27.2	30.3	26.9	15.0	1	
M	296	26.7	26.7	23.3	23.3	1.2 (0.95–1.6)	
HIV status							1.0
Negative	141	26.2	30.5	23.4	19.9	1	
Positive	288	26.4	28.1	27.8	17.7	1.0 (0.7–1.4)	
Area							0.005
South, near Chilumba	70	38.6	22.9	24.3	14.3	0.51 (0.31–0.84)	
Middle, near Nyungwe	89	20.2	22.5	30.3	27.0	1.2 (0.79–1.9)	
Around Karonga	119	25.2	34.5	21.9	18.5	0.73 (0.48–1.1)	
Karonga central (urban)	209	21.5	25.4	32.5	20.6	1	
Kaporo area	84	35.7	34.5	15.5	14.3	0.46 (0.29–1.1)	
Far north	57	28.1	40.4	21.1	10.5	0.54 (0.32–0.9)	
Outside district	16	26.9	28.9	25.6	18.6	0.41 (0.61–1.1)	
Tuberculosis type							0.6
Smear positive	485	26.2	29.5	25.4	19.0	1	
Smear negative	145	29.7	24.8	27.6	17.9	0.94 (0.68–1.3)	
Extrapulmonary	52	30.8	32.7	19.2	17.3	0.78 (0.47–1.3)	
Previous tuberculosis							0.6
No	628	27.4	28.8	25.8	18.0	1	
Yes	47	27.7	25.5	21.3	25.5	1.2 (0.68–2.0)	
Isoniazid resistance							0.2
No	641	28.1	27.9	25.9	18.1	1	
Yes	39	12.8	43.6	15.4	28.2	1.5 (0.84–2.6)	
Died†							1.0
No	382	26.7	28.3	25.1	19.9	1	
Yes	155	25.8	29.0	26.5	18.7	1.0 (0.72–1.4)	

*Odds ratio from ordered logistic regression. This represents the summary relative odds of larger clusters compared with smaller clusters across the 4 groups. Odds ratios >1 imply that the odds of being in a larger cluster are greater than in the baseline group. CI, confidence interval.
†Outcome recorded for 632 patients; those who were lost or transferred while receiving treatment are excluded from this analysis.

Table 2. Multivariate analysis of risk factors for larger cluster size, Malawi

Risk factor	Cluster size*		p value
	Odds ratio	95% CI	
Age, y			
<25	1		0.01†
25–34	1.0	0.66–1.5	
35–44	0.76	0.48–1.2	
≥45	0.62	0.39–1.0	
Male	1.4	1.0–1.8	0.03
Area			<0.001
South, near Chilumba	0.54	0.33–0.90	
Middle, near Nyungwe	1.3	0.81–2.0	
Around Karonga	0.74	0.50–1.1	
Karonga central (urban)	1		
Kaporo area	0.48	0.30–0.76	
Far north	0.55	0.32–0.92	
Outside district	0.41	0.15–1.1	

*Odds ratios are adjusted for the other factors shown in the table (i.e., the multivariate equation contained age group, sex, and area). CI, confidence interval.
†Trend.

2002. Thirty-six (29%) had spoligotype 59, and 10 more had closely related patterns; 11 (9%) had spoligotype 21; 8 (6%) had spoligotype 53, and 2 had closely related patterns; 7 (6%) had the Beijing spoligotype; and 8 (6%) had spoligotype 129. The 36 isolates with spoligotype 59 had 23 different RFLP patterns with a similarity coefficient of 63%.

Discussion

This study suggests that both epidemiologic and strain-related factors may contribute to large cluster size. In large clusters young adults, male patients, and those living in the town were over-represented, all factors likely to be associated with increased social mixing. Similar associations with age and sex have been found previously, in the United States and Denmark. In Denmark the largest cluster was particularly predominant in the capital city (*1*,*2*).

There was no significant association between tuberculosis type (smear positive, smear-negative pulmonary, or extrapulmonary) and cluster size, but most patients had sputum smear–positive disease. There was also no statistically significant association with degree of smear positivity (not shown). An overall association with infectiousness would not necessarily be expected: the infectiousness of the first cases of a cluster may be important in determining size, but the first cases for the large clusters, which were found throughout the period of study, are not identifiable. There was no significant association with

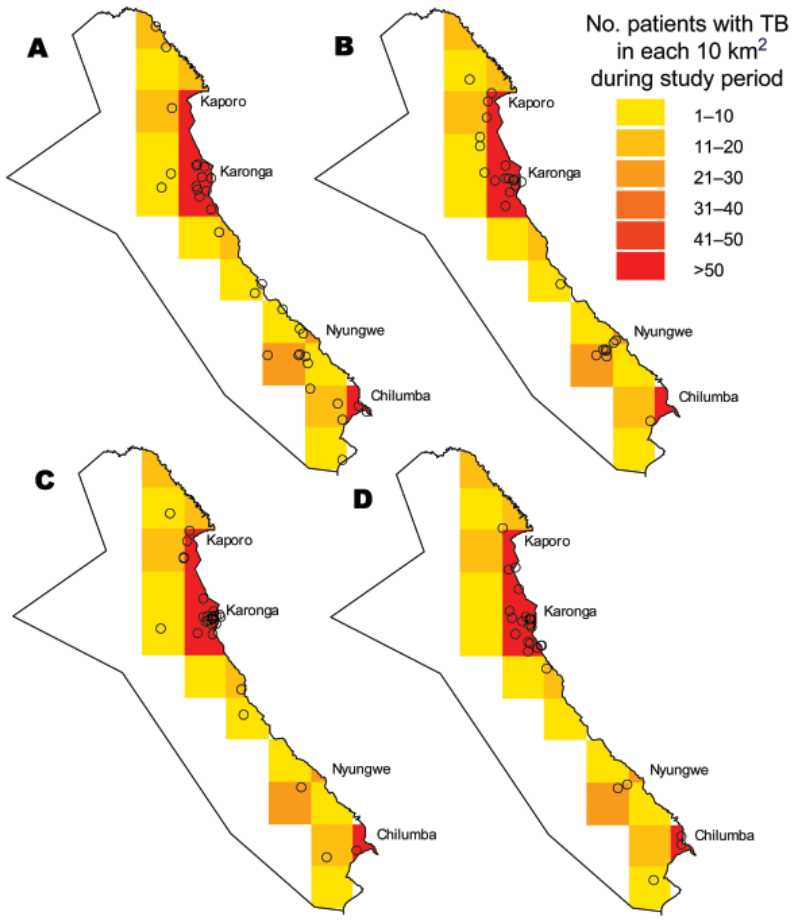


Figure. Geographic distribution of the 4 most common strains defined by restriction fragment length polymorphism: A) strain kps12, B) strain kps121, C) strain kps41, and D) strain kps44. Each o represents a patient. Each square is 10 km × 10 km. The background shading represents the total number of tuberculosis (TB) cases in each area during the study period, which largely reflects the population density.

Table 3. Proportion of all tuberculosis cases caused by each of the RFLP-defined large cluster strains over time, Malawi*

Strain	No. patients	Tuberculosis cases caused by each strain, % (no.)				p value
		1995–1997	1998–1999	2000–2001	2002–2003	
Kps10	18	2.3 (6)	0.9 (3)	1.7 (5)	2.8 (4)	0.4
Kps12	34	1.5 (4)	3.9 (13)	4.9 (14)	2.1 (3)	0.1
Kps20	15	1.9 (5)	0.9 (3)	1.7 (5)	1.4 (2)	0.7
Kps21	15	0.8 (2)	2.1 (7)	2.1 (6)	0.0 (0)	0.2
Kps41	37	4.2 (11)	3.0 (10)	4.5 (13)	2.1 (3)	0.5
Kps44	29	3.8 (10)	3.3 (11)	1.4 (4)	2.8 (4)	0.3
Kps64	16	0.4 (1)	1.5 (5)	2.8 (8)	1.4 (2)	0.1
Kps97	15	0.8 (2)	2.4 (8)	1.0 (3)	0.7 (1)	0.3
Kps104	16	1.5 (4)	0.9 (3)	2.1 (6)	2.1 (3)	0.6
Kps121	27	4.2 (11)	2.7 (9)	2.1 (6)	0.7 (1)	0.03 (trend)

*RFLP, restriction fragment length polymorphism.

isoniazid resistance, but only 39 (6%) patients had resistant strains. Isoniazid resistance has been associated with reduced clustering and reduced generation of secondary cases (*15*,*16*) so it might have been expected to be less common in the larger clusters. Only 3 clustered patients had rifampin resistance in our study (2 with 1 strain and 1 with another), so the effect of this factor on cluster size could not be investigated.

The factors associated with cluster size were not identical to those associated with clustering overall (*5*). Whereas younger adults were more likely to have clustered strains and to be in large clusters, female patients were more likely to have clustered strains but among clustered case-patients, male patients were more likely to be in large clusters. Known contact with a previous tuberculosis patient is an important risk factor for tuberculosis, especially for women in this population (*17*). It may be that women are particularly likely to become infected at home (and therefore be in small clusters) and that men are more likely to become infected outside the home, sometimes from outside the area (seen as unique strains) and sometimes as part of large clusters.

We found no evidence of an association of cluster size with HIV status, although we had previously found HIV to be associated with clustering among older patients (*5*). The effect of HIV infection on clustering is complex since it depends both on the biologic effects of HIV (increasing the risks for active disease—perhaps to different extents for primary and postprimary disease—and decreasing infectiousness) and on any tendency for HIV and tuberculosis to affect the same subpopulations with shared risk factors.

Strain virulence was assessed by examining the proportion of patients who died: there was no association with cluster size either overall, or separately, in HIV-positive or -negative patients (data not shown). Virulent strains could lead to large clusters if virulence were associated with increased transmission rates or increased rates of disease after infection (*3*). However, virulent strains could have less opportunity to transmit if the severity of symptoms leads to early treatment or death, thus reducing the duration of the infectious period.

Evidence that strain characteristics may have contributed to cluster size comes from the finding that the spoligotypes of most of the common RFLP-defined strains in this study were identical to, or only 1 spacer different from, widespread spoligotypes already described. Unique RFLP-defined strains from smear-positive patients in the early part of the study were used as a comparison group.

Table 4. Spoligotypes for the RFLP-defined large cluster strains with at least 5 bands*

Strain no.	No. bands on RFLP	No. examples spoligotyped	Spoligotype octal description	International classification	Comment
Kps41	11	5	777777606060771	59	Widespread
Kps20	8	3	777777606060771	59	Widespread
Kps21	8	2	777777606060771	59	Widespread
Kps10	10	1	777777606060771	59	Widespread
		1	777777206060771	Not recorded	
Kps12	9	3	577777606060771	Not recorded	
Kps64	9	2	777777606060771	59	Widespread
		1	777437606060731	Not recorded	
Kps121	13	2	700777747413771	129	3 recorded, Zimbabwe, French Guiana
Kps104	14	2	703377400001771	21	Widespread
		1	703377400001631	Not recorded	
Kps44	16	5	777777777760771	53	Widespread
Kps97	22	2	000000000003771	1	Widespread, Beijing

*RFLP, restriction fragment length polymorphism.

Smear-positive case-patients were chosen to maximize the likelihood of transmission occurring; early cases were used to allow time for secondary cases to have been identified if they had occurred. These unique strains were less likely than the large cluster strains to have spoligotypes that were closely related to widespread types, but this difference was not statistically significant, and the spoligotypes that were found in the large cluster strains were also found among the unique strains. Interestingly, strain kps121, which was the only large cluster strain with a spoligotype not closely related to a widespread previously described type, was also the 1 large cluster strain that was clearly decreasing in the Karonga population.

The finding of large cluster strains with previously described widespread spoligotypes may suggest that these strains are particularly transmissible or particularly likely to cause disease. Other possibilities are that they are older in evolutionary terms, and thus have had more time to become widespread, or that we are seeing a founder effect in some populations with subsequent spread following human migration patterns. Spoligotype 59 was common in the Malawi population in all groups of patients, clustered and unique, and was associated with a wide diversity of RFLP patterns, which suggests that it may be a longstanding strain in this area. It was also the most common spoligotype found in studies in Zimbabwe and Zambia (*18*,*19*). However, spoligotype 59 was particularly common among the isolates from large clusters, with more closely related RFLP patterns, consistent with some variants having high transmissibility. Spoligotype 59 has been classified as belonging to the Latin-American-Mediterranean lineage (*18*), and as part of the strain family Southern Africa Family 1 (*19*). The large cluster strain kps97 had a Beijing spoligotype and in total, we have previously identified 44 patients with Beijing strains in this dataset, with 12 different RFLP patterns (*14*). Beijing strains have been associated with increased virulence and growth rates in vitro (*20*–*22*). That there are true differences in strain characteristics between other clustered and nonclustered strains is beginning to be established in in vitro studies from other populations (*23*).

Acknowledgments

We thank the Government of the Republic of Malawi for their interest in this project and the National Health Sciences Research Committee of Malawi for permission to publish the article. We also thank Pam Sonnenberg for helpful comments on an earlier draft, Keith Branson for the maps, and Sian Floyd for statistical advice.

Until 1996 the Karonga Prevention Study was funded primarily by the British Leprosy Relief Association and the International Federation of Anti-Leprosy Organizations, with contributions from the World Health Organization/United Nations Development Program/World Bank Special Programme for Research and Training in Tropical Diseases. Since 1996, the Wellcome Trust has been the principal funder. J.R.G. was supported in part by the UK Department for International Development and the UK Department of Health (Public Health Career Scientist award).

Dr Glynn is professor of infectious disease epidemiology at the London School of Hygiene and Tropical Medicine, London, UK. Her research interests include tuberculosis, HIV, and molecular epidemiology.

References

1. Giordano TP, Soini H, Teeter LD, Adams GJ, Musser JM, Graviss EA. Relating the size of molecularly defined clusters of tuberculosis to the duration of symptoms. Clin Infect Dis. 2004;38:10–6. DOI: 10.1086/380454
2. Lillebaek T, Dirksen A, Kok-Jensen A, Andersen AB. A dominant *Mycobacterium tuberculosis* strain emerging in Denmark. Int J Tuberc Lung Dis. 2004;8:1001–6.
3. Valway SE, Sanchez MP, Shinnick TF, Orme I, Agerton T, Hoy D, et al. An outbreak involving extensive transmission of a virulent strain of *Mycobacterium tuberculosis.* N Engl J Med. 1998;338:633–9. DOI: 10.1056/NEJM199803053381001
4. Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. Trends Microbiol. 2002;10:45–52. DOI: 10.1016/S0966-842X(01)02277-6
5. Glynn JR, Crampin AC, Yates MD, Traore H, Mwaungulu FD, Ngwira BM, et al. The importance of recent infection with *M. tuberculosis* in an area with high HIV prevalence: a long-term molecular epidemiological study in northern Malawi. J Infect Dis. 2005;192:480–7. DOI: 10.1086/431517
6. Glynn JR, Crampin AC, Ngwira BM, Mwaungulu FD, Mwafulirwa DT, Floyd S, et al. Trends in tuberculosis and the influence of HIV infection in northern Malawi, 1988–2001. AIDS. 2004;18:1459–63. DOI: 10.1097/01.aids.0000131336.15301.06
7. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993;31:406–9.
8. Glynn JR, Yates MD, Crampin AC, Ngwira BM, Mwaungulu FD, Black GF, et al. DNA fingerprint changes in tuberculosis: re-infection, evolution, or laboratory error? J Infect Dis. 2004;190:1158–66. DOI: 10.1086/423144
9. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. 1997;35:907–14.
10. Filliol I, Driscoll JR, Van Soolingen D, Kreiswirth BN, Kremer K, Valetudie G, et al. Global distribution of *Mycobacterium tuberculosis* spoligotypes. Emerg Infect Dis. 2002;8:1347–9.
11. Filliol I, Driscoll JR, van Soolingen D, Kreiswirth BN, Kremer K, Valetudie G, et al. Snapshot of moving and expanding clones of *Mycobacterium tuberculosis* and their global distribution assessed by spoligotyping in an international study. J Clin Microbiol. 2003;41:1963–70. DOI: 10.1128/JCM.41.5.1963-1970.2003
12. StataCorp. STATA base reference manual. College Station (TX): STATA Press; 2003.
13. Dale JW, Brittain D, Cataldi AA, Cousins D, Crawford JT, Driscoll J, et al. Spacer oligonucleotide typing of bacteria of the *Mycobacterium tuberculosis* complex: recommendations for standardised nomenclature. Int J Tuberc Lung Dis. 2001;5:216–9.

14. Glynn JR, Crampin AC, Traore H, Yates MD, Mwaungulu F, Ngwira B, et al. *Mycobacterium tuberculosis* Beijing genotype, northern Malawi. Emerg Infect Dis. 2005;11:150–3.
15. van Soolingen D, Borgdorff MW, de Haas PE, Sebek MM, Veen J, Dessens M, et al. Molecular epidemiology of tuberculosis in the Netherlands: a nationwide study from 1993 through 1997. J Infect Dis. 1999;180:726–36. DOI: 10.1086/314930
16. Burgos M, DeRiemer K, Small PM, Hopewell PC, Daley CL. Effect of drug resistance on the generation of secondary cases of tuberculosis. J Infect Dis. 2003;188:1878–84. DOI: 10.1086/379895
17. Crampin AC, Glynn JR, Floyd S, Malema SS, Mwinuka VM, Ngwira B, et al. Tuberculosis and gender: exploring the patterns in a case control study in Malawi. Int J Tuberc Lung Dis. 2004;8:194–203.
18. Easterbrook PJ, Gibson A, Murad S, Lamprecht D, Ives N, Ferguson A, et al. High rates of clustering of tuberculosis strains in Harare, Zimbabwe: a molecular epidemiological study. J Clin Microbiol. 2004;42:4536–44. DOI: 10.1128/JCM.42.10.4536-4544.2004
19. Chihota V, Apers L, Mungofa S, Kasongo W, Nyoni IM, Tembwe R, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in regions of Southern Africa. Int J Tuberc Lung Dis. 2007;11:311–8.
20. Lopez B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, et al. A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol. 2003;133:30–7. DOI: 10.1046/j.1365-2249.2003.02171.x
21. Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, Roholl P, et al. Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different *Mycobacterium tuberculosis* genotypes in a BALB/c mouse model. Clin Exp Immunol. 2004;137:460–8. DOI: 10.1111/j.1365-2249.2004.02551.x
22. Zhang M, Gong J, Yang Z, Samten B, Cave MD, Barnes PF. Enhanced capacity of a widespread strain of *Mycobacterium tuberculosis* to grow in human macrophages. J Infect Dis. 1999;179:1213–7. DOI: 10.1086/314738
23. Theus SA, Cave MD, Eisenach KD. Intracellular macrophage growth rates and cytokine profiles of *Mycobacterium tuberculosis* strains with different transmission dynamics. J Infect Dis. 2005;191:453–60. DOI: 10.1086/425936

Address for correspondence: Judith R. Glynn, Department of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, Keppel St, London WC1E 7HT, UK; email: judith.glynn@lshtm.ac.uk

Spotted Fever Group Rickettsiae in Ticks, Morocco

Mhammed Sarih,*[1] Cristina Socolovschi,†[1] Najma Boudebouch,* Mohammed Hassar,* Didier Raoult,† and Philippe Parola†

A total of 370 ticks, encompassing 7 species from 4 genera, were collected during 2002–2006 from domestic animals and vegetation in the Taza region of northeastern Morocco. Rickettsial DNA was identified in 101 ticks (27%) by sequencing PCR products of fragments of the citrate synthase and outer membrane protein genes of *Rickettsia* spp. Seven rickettsiae of the spotted fever group were identified, including 4 pathogens: *R. aeschlimannii* in *Hyalomma marginatum marginatum*, *R. massiliae* in *Rhipicephalus sanguineus*, *R. slovaca* in *Dermacentor marginatus*, and *R. monacensis* in *Ixodes ricinus.* Two suspected pathogens were also detected (*R. raoultii* in *D. marginatus* and *R. helvetica* in *I. ricinus)*. An incompletely described *Rickettsia* sp. was detected in *Haemaphysalis* spp. ticks.

Tick-borne rickettsioses are infections caused by obligate intracellular gram-negative bacteria of the spotted fever group (SFG) in the genus *Rickettsia* and the order Rickettsiales. These zoonoses are now recognized as emerging vector-borne infections worldwide (*1*,*2*). They share characteristic clinical features, including fever, headache, rash, and occasional eschar formation at the site of the tick bite. Although these diseases have been known for a long time, they have been poorly investigated in northern Africa, including Morocco (*2*).

Two human tick-borne SFG rickettsioses are known to occur in Morocco. Mediterranean spotted fever, caused by *Rickettsia conorii conorii*, is transmitted by the brown dog tick, *Rhipicephalus sanguineus*, which is well adapted to urban environments and is endemic to the Mediterranean area (*2*). In Morocco, clinicians usually consider patients with spotted fever as having Mediterranean spotted fever. However, in 1997, Beati et al. isolated a new rickettsia, *R. aeschlimannii,* from *Hyalomma marginatum marginatum* ticks collected in Morocco (*3*). In 2002, human infection with this rickettsia was reported in a patient returning from Morocco to France (*4*).

To date, all studies on rickettsioses conducted in Morocco have been based on only clinical and serologic features. However, the number of representatives of the genus *Rickettsia* and the number of newly described rickettsioses have increased in recent decades because of improved cell culture isolation techniques and extensive use of bacterial detection and identification by molecular biologic techniques (*2*). Comparison of the sequences of PCR-amplified fragments of genes encoding 16S rRNA, citrate synthase (*glt*A), or outer membrane protein (*ompA*) has become a reliable method for identifying rickettsiae in arthropods, including ticks (*1*). Therefore, our aim was to detect and characterize rickettsiae in hard ticks collected in Morocco by using PCR and sequence analysis of amplified products and to discuss their potential threat for humans and animals.

Materials and Methods

Collection and Identification of Ticks

From April 2002 through March 2006, ticks were collected from domestic animals (livestock and dogs) and by flagging vegetation at sites in the Taza region in northeastern Morocco. These sites were located between the towns of Babboudir and Babezhare, (34°12′48.81″N, 4°0′55.63″W) in the Atlas Mountains, situated 40 km from the city of Taza and 90 km from the city of Fez. All ticks collected were adults and morphologically identified to the

*Institut Pasteur du Maroc, Casablanca, Morocco; and †World Health Organization Collaborative Center for Rickettsioses and Other Arthropod Borne Bacterial Diseases, Marseille, France

DOI: 10.3201/eid1407.070096

[1]These authors contributed equally to this article.

species or genus level by using standard taxonomic keys. Ticks were kept in ethanol at room temperature until DNA was extracted in the Laboratoire des Maladies Vectorielles, Institut Pasteur du Maroc, Casablanca, Morocco. DNA samples were thereafter sent to the Unité des Rickettsies in Marseille, France.

PCR Detection and Identification of *Rickettsia* spp.

Ticks were rinsed with distilled water for 10 min, dried on sterile filter paper in a laminar flow hood, and crushed individually in sterile Eppendorf (Hamburg, Germany) tubes. DNA was extracted by using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Rickettsial DNA was detected by PCR by using primers *Rp* CS.409p and *Rp* CS.1258n (Eurogentec, Seraing, Belgium), which amplify a 750-bp fragment of the *gltA* gene of *Rickettsia* spp. as described (*5*). All ticks positive for *gltA* were tested for the *ompA* gene of *Rickettsia* spp. by using primers Rr. 190.70 and Rr. 190.701, which amplify a 629–632-bp fragment (*5*). A negative control (distilled water instead of tick DNA template) and a positive control (DNA from *R. montanensis*) were included in each test. All PCRs were conducted in Marseille by using the GeneAmp PCR System 2400 and 9700 thermal cyclers (PerkinElmer, Waltham, MA, USA). Amplification products were analyzed after electrophoresis on a 1% agarose gel stained with ethidium bromide. To identify detected *Rickettsia* spp., PCR products were purified and sequencing was performed as described (*5*). All sequences obtained were assembled and edited with Auto Assembler software version 1.4 (PerkinElmer). Sequences were analyzed by BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) sequencing analysis of sequences in the GenBank database.

Molecular Identification of Ticks

To help identify the ticks at the species level, molecular tools were used for some ticks that had not been morphologically identified at the species level and that were positive for rickettsiae. Amplification by PCR with T1B and T2A primers and sequencing of a 338-bp amplified fragment of the 12S rRNA gene of the ticks were performed as described (*6*).

Results

A total of 370 specimens representing 7 species and 4 genera of ticks were collected. Tick species identified by taxonomic keys included *Rh. sanguineus* (106 specimens), *Rh. bursa* (76), *Rh. turanicus* (25), *Haemaphysalis sulcata* (79), *Ha. punctata* (6), *Ixodes ricinus* (14), and *Dermacentor marginatus* (11) (Figure). Some ticks, including engorged females or damaged specimens, were identified to genus only (18 *Haemaphysalis* sp. and 35 *Hyalomma* sp.). Most ticks (337) were collected from domestic animals; the rest were collected by flagging of vegetation (Table).

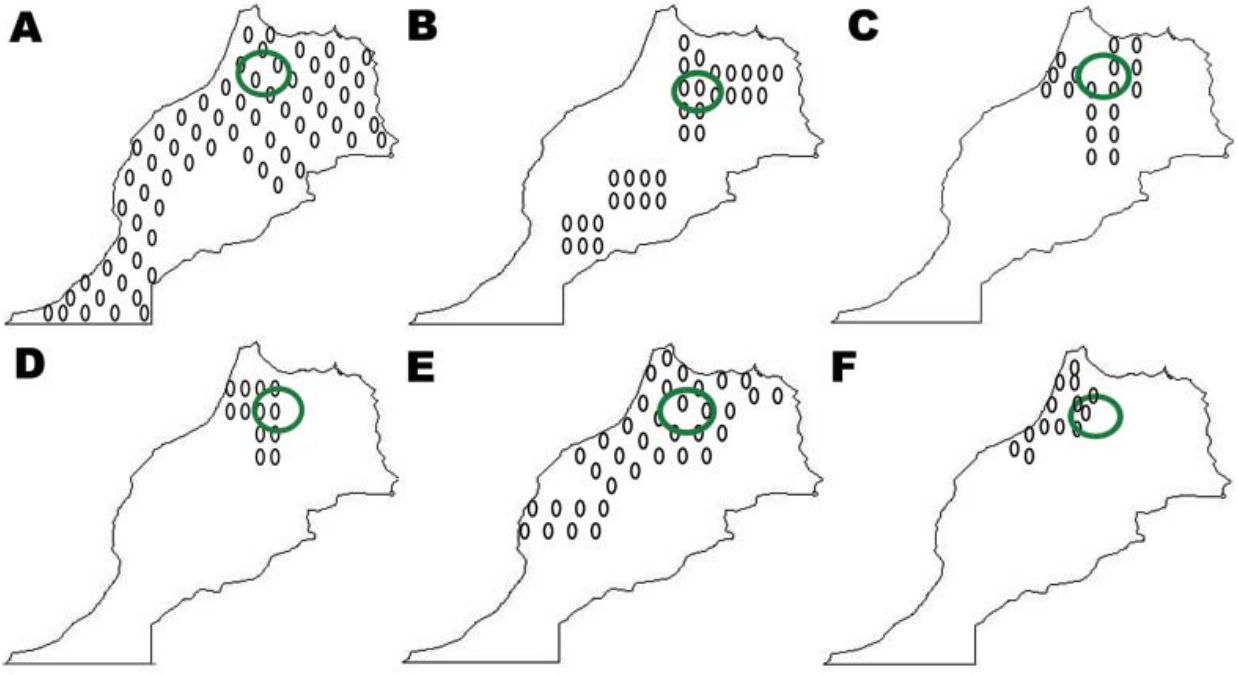


Figure. Distribution of ticks in Morocco from which rickettsial DNA was detected by PCR. A) *Rhipicephalus sanguineus*, B) *Haemaphysalis sulcata*, C) *Ha. punctata*, D) *Ixodes ricinus*, E) *Hyalomma marginatum marginatum*, F) *Dermacentor marginatus.* Green circles indicate areas where ticks were collected and found to harbor rickettsiae. Ovals indicate distribution of each tick species in which rickettsial DNA was detected by PCR.

Rickettsial DNA was detected in 101 (27%) of 370 ticks by using a *glt*A PCR. Three (8.6%) of 35 *Hyalomma* spp. ticks contained rickettsia DNA with a *glt*A gene fragment that was 99.1% (765/772 bp) similar to that of *R. aeschlimannii* and 100% similar to the *omp*A gene of *R. aeschlimannii*. A 237-bp fragment of tick mitochondrial 12S rDNA gene was obtained from one of the *R. aeschlimannii*–infected ticks. The sequence of this fragment enabled definitive identification of the tick to the species level, with 100% similarity to *H. marginatum marginatum* (GenBank accession no. AF150034).

Five (4.7%) of 106 *Rh. sanguineus* ticks were positive for rickettsial DNA by PCR. For all samples, sequence analyses showed 99.8% (636/637) similarity with the *glt*A sequence and 100% similarity with the *omp*A sequence of *R. massiliae*. One *R. massiliae*–infected tick was evaluated by PCR amplification of the tick mitochondrial 12S rDNA gene; sequence analyses showed 99.6% (235/236) similarity to the corresponding 12S rDNA of *Rh. sanguineus* (GenBank accession no. AF133056).

A total of 5 (45.5%) of 11 *D. marginatus* ticks contained a rickettsia with a nucleotide sequence of *glt*A that was 99.2% (635/640 bp) similar to *R. slovaca* and 100% (533/533 bp) similar to the *omp*A sequence of *R. slovaca*. Rickettsial DNA was detected in 1 other specimen of *D. marginatus*. Amplified *glt*A and *omp*A fragments were sequenced and showed 99.3% (560/564 bp) similarity with the *glt*A gene of *R. raoultii* and 100% similarity with the *omp*A gene of *R. raoultii*.

Five (35.7%) of 14 specimens of *I. ricinus* were positive by *glt*A PCR. Sequence analyses showed 100% homology with the corresponding *glt*A sequence of *R. monacensis*. *Omp*A sequences were obtained and showed 99.7% (585/587 bp) similarity with the corresponding sequence of *R. monacensis*. Four (28.6%) of 14 *I. ricinus* ticks contained rickettsia with nucleotide sequences of *glt*A with 99.8% (633/634 bp) similarity to *R. helvetica*. The primer set Rr.190.70p-Rr.190.701n failed to amplify an *omp*A product in any specimens that were positive for the *glt*A gene of *R. helvetica.*

Sixty-one (77.2%) of 79 *Ha. sulcata* ticks, 3 (50%) of 6 *Ha. punctata* ticks, and 14 (77.7%) of 18 *Haemaphysalis* spp. ticks were positive by PCR for the primer set *Rp* CS.409p and *Rp* CS.1258n for the *glt*A gene. The *glt*A sequences obtained were different from all known *Rickettsia* spp. sequences deposited in GenBank. The most closely related sequence of *glt*A was designated "Ricketttsia endosymbiont of *Haemaphysalis sulctata*" (99.4% similarity; 484/487 bp). The next most closely related sequence of *glt*A, with 96% similarity, was *R. felis*. Results of the PCR with the *omp*A primer set Rr.190.70p-Rr.190.701n were negative for all *Haemaphysalis* spp. ticks that were positive for the *glt*A gene.

Table. Detection and identification of spotted fever group *Rickettsia* spp. from ticks collected in Morocco, by PCR and DNA sequencing

Tick species (no. specimens tested)	Host	Rickettsial gene targeted/no. ticks positive by PCR/no. examined*	Identification by gene sequence (no. identified/no. tested)	GenBank accession no.
Dermacentor marginatus (11)	Vegetation	*glt*A/6/11	*R. slovaca* (5/6), *R. raoultii* (1/6)	U59725, DQ365803
		*omp*A/6/6	*R. slovaca* (5/6), *R. raoultii* (1/6)	U43808, DQ365799
Hyalomma marginatum marginatum (35)	Domestic animals	*glt*A/3/35	*R. aeschlimannii*	U59722
		*omp*A/3/3	*R. aeschlimannii*	DQ379982
Haemaphysalis sulctata (79)	Domestic animals	*glt*A/61/79	*"Rickettsia endosymbiont of Ha. Sulctata" ("R. kastelanii")*	DQ081187
		*omp*A/0/61	–	–
Ha. punctata (6)	Domestic animals	*glt*A/3/6	*"Rickettsia endosymbiont of Ha. Sulctata" ("R. kastelanii")*	DQ081187
		*omp*A/0/3		
Haemaphysalis sp. (18)	Vegetation	*glt*A/14/18	*"Rickettsia endosymbiont of Ha. Sulctata" ("R. kastelanii")*	DQ081187
		*omp*A/0/14	–	–
Ixodes ricinus (14)	Vegetation	*glt*A/9/14	*R. monacensis* (5/9), *R. helvetica* (4/9)	AF140706, U59723
		*omp*A/5/9	*R. monacensis* (5/9)	AJ427885
Rhipicephalus sanguineus (106)	Domestic animals	*glt*A/5/106	*R. massiliae*	U59720
		*omp*A/5/5	*R. massiliae*	U43792
Rh. bursa (76)	Domestic animals	*glt*A/0/76	–	–
Rh. turanicus (25)	Domestic animals	*glt*A/0/25	–	–

*_glt_A, citrate synthase A; _omp_A, outer membrane protein A. Only ticks positive for the _glt_A gene were tested for the _omp_A gene.

None of the *Rh. bursa* and *Rh. turanicus* ticks harbored rickettsiae. All GenBank accession numbers used to compare sequences obtained from ticks are shown in the Table.

Discussion

Before this study, only 2 SFG rickettsiae pathogenic to humans had been described in Morocco, *R. conorii conorii*, the agent of Mediterranean spotted fever, and the recently described *R. aeschlimannii* (*2*,*3*). In our study, in addition to *R. aeschlimannii,* we identified 3 other SFG pathogenic rickettsiae in Morocco: *R. massiliae*, *R. slovaca*, and *R. monacensis*. Furthermore, 2 tick-borne SFG *Rickettsia* spp. presumptively associated with human illnesses, *R. helvetica* and *R. raoultii*, and an undescribed bacterium have been identified.

DNA extraction and PCR were performed in different locations (Morocco and France), and all results were supported by 2 sets of primers. The *gltA* primers used in the first screening are known to amplify all known tick-borne rickettsiae (*7*). A second set of primers targeting the *ompA* gene was used to confirm positive results, although some rickettsia (e.g., *R. helvetica*) cannot be amplified by using this set. There were no cases in which multiple species of rickettsiae were detected in an infected tick, as in most of the similar molecular surveys published (*1*,*2*). Our results did not address prevalence and distribution of rickettsiae detected. Systematic sampling was not conducted. Also, some tick samples tested with rickettsial primers have not been tested with tick primers in parallel. Therefore, inhibitors that could be responsible for false-negative results and underestimation of infection rates cannot be ruled out.

R. aeschlimannii was isolated from *H. marginatum marginatum* ticks collected in Morocco in 1997 (*3*). This rickettsia has also been detected in *H. marginatum rufipes* ticks in Zimbabwe, Niger, and Mali; in *H. marginatum marginatum* in Portugal, Croatia, Spain, Greece, Algeria, and Egypt; and in both ticks in Corsica (*2*,*8*,*9*). *H. marginatum marginatum* is also known as the Mediterranean *Hyalomma* and may represent up to 42% of ticks found on cattle in Morocco. This tick is also a suspected reservoir of *R. aeschlimannii* because transstadial and transovarial transmission have been reported (*8*). As a result, the distribution of *R. aeschlimannii* may parallel that of *H. marginatum marginatum*.

In 2002, the pathogenic role of infection with *R. aeschlimannii* was demonstrated by PCR and serologic testing in a patient who returned to France from Morocco (*4*). Clinical signs in this 36-year-old man were fever, generalized maculopapular rashes, and a vesicular lesion of the ankle that became necrotic and resembled the typical tache noire of Mediterranean spotted fever. A second case was identified in a patient in South Africa in 2002 (*10*). This patient had an eschar around the attachment site. No additional symptoms developed, and treatment with antimicrobial drugs may have prevented progression of the syndrome.

A total of 4.7% of the *Rh. sanguineus* ticks tested were infected by *R. massiliae*. This rickettsia was isolated from *Rh. sanguineus* ticks collected near Marseille, France, in 1992 (*11*). It has been also found in *Rh. sanguineus* and *Rh. turanicus* in Greece, Spain, Portugal, Switzerland, central Africa, and Mali (*2*,*12*,*13*). Eremeeva et al. (*14*) recently reported detection and isolation of *R. massiliae* from 2 of 20 *Rh. sanguineus* ticks collected in eastern Arizona in the United States. *R. massiliae* may be commonly associated with these ticks, which are distributed worldwide. Transstadial and transovarial transmission of rickettsia in ticks has been reported (*13*).

In 2003, serologic findings from Spain showed that in 5 of 8 serum samples titers against *R. massiliae* were higher than those against *R. conorii*, the agent of Mediterranean spotted fever (*12*). The authors analyzed clinical symptoms of patients with strong serologic reactions against *R. massiliae* antigens but did not find relevant clinical differences between these patients and those with Mediterranean spotted fever. However, it is generally recognized that there are relatively few clinical differences among the different spotted fever diseases, and these differences are occasionally not taken into account by clinicians when reporting clinical data of patients (*12*). The only confirmed case of a person infected with *R. massiliae* was a patient hospitalized in Sicily, Italy. This patient had fever, a maculopapular rash on the palms of his hands and the soles of his feet, an eschar, and hepatomegaly. The strain of *R. massiliae* was isolated in Vero cells in 1985 and stored for 20 years in Sicily, but was not definitively identified until 2005 at the Unité de Rickettsies in Marseille, France (*15*).

The third SFG pathogenic rickettsia found in our study was *R. slovaca* in 5 (45.5%) of 11 *D. marginatus*. *R. slovaca,* which was identified in *Dermacentor* spp. ticks in Slovakia in 1968, has been subsequently found in *D. marginatus* and *D. reticulatus* in France, Switzerland, Portugal, Spain, Armenia, Poland, Bulgaria, Croatia, Russia, and Germany (*2*,*16*). These ticks may act as vectors and reservoirs of *R. slovaca,* which is maintained in ticks through transstadial and transovarial transmission (*17*). Human infection with *R. slovaca* was reported in France in 1997. Patients with similar clinical signs were observed in Spain, Bulgaria, and Hungary, where the syndrome was known as tick-borne lymphadenopathy or *Dermacentor*-borne necrosis erythema lymphadenopathy because of eschar at the tick bite site in the scalp and cervical lymphadenopathy (*2*,*18*–*20*). The incubation period ranges from 4 to 15 days. Low-grade fever and rash were present. The acute disease can be followed by fatigue and residual alopecia at the bite site (*16*,*21*). Recently, Gouriet et al. reported 14 new cases

with tick-borne lymphadenopathy and *Dermacentor*-borne necrosis erythema lymphadenopathy in southern France during January 2004–May 2005 (*22*). In this group, tick-borne lymphadenopathy occurred mainly in young children and women and during the colder months (*22*). Overall, data in our study indicate that clinicians should be aware that this tick-related disorder may be found in Morocco.

R. raoultii is a recently described SFG rickettsia (*23*). In 1999, three new rickettsial genotypes, RpA4, DnS14, and DnS28, were identified in ticks collected in Russia by using PCR amplification and sequencing of 16S rDNA, *gltA*, and *ompA* genes. Genotypes identical to DnS14, DnS28, and RpA4 were thereafter detected in various areas in Russia and Kazakhstan in *D. reticulatus*, *D. marginatus*, and *D. silvarum* (*24*), in Germany and Poland in *D. reticulatus* (*25*,*26*), and in Spain, France, and Croatia in *D. marginatus* (*23*). Recently, cultivation of 2 rickettsial isolates genetically identical to *Rickettsia* sp. genotype DnS14, two rickettsial isolates genetically identical to *Rickettsia* sp. genotype RpA4, and 1 rickettsial isolate genetically identical to *Rickettsia* sp. genotype DnS28 was described (*23*). These isolates have been shown to fulfill the requirements for their classification within a new species, *R. raoultii*, by using multigene sequencing (16S rDNA, *gltA*, *ompA*, *ompB*, *sca4*, *ftsY*, and *rpoB* genes) and serotyping techniques (*23*,*27*). In our study, we detected *R. raoultii* in *D. marginatus* in Morocco. This tick is found in the cooler and more humid areas of the Mediterranean region associated with the Atlas Mountains. It is restricted to small areas of Morocco and Tunisia (*28*). Detection of *R. raoultii* in Morocco is of clinical relevance because it is suspected to be a human pathogen. In 2002, it was detected in *D. marginatus* obtained from a patient in France in whom typical clinical symptoms of tick-borne lymphadenopathy developed (*23*).

R. helvetica is another species identified in Morocco in this study. It is one of the few SFG species in which a commonly used o*mp*A primer set does not amplify a PCR product (*7*,*29*). However, sequencing *glt*A enabled definitive identification. *R. helvetica* was isolated in Switzerland from *I. ricinus* in 1979 and has been identified in many European countries, where the tick is both a vector and a reservoir (*2*). The distribution of *R. helvetica* is not limited to Europe but extends into Asia (*30*). Our data show that the distribution of this bacterium extends into northern Africa. A small population of *I. ricinus* is present in Tunisia, Algeria, and Morocco. Our study was conducted in Taza, a humid area in the middle of the Atlas Mountains, which was the only site in Morocco that contained *I. ricinus* ticks (*2*).

R. helvetica was considered to be a nonpathogenic rickettsia for ≈20 years after its discovery. However, in 1999 it was implicated in fatal perimyocarditis in patients in Sweden (*31*). The authors of this study subsequently reported a controversial association between *R. helvetica* and sarcoidosis in Sweden (*32*) and found *R. helvetica* DNA in human aortic valves (*33*). However, the validity of these associations has been questioned by some rickettsiologists (*2*), and additional studies did not detect antibodies to rickettsia in a group of Scandinavian sarcoidosis patients (*34*). In 2000, seroconversion for *R. helvetica* was described in a patient in France with a nonspecific febrile illness (*35*). Serologic data, including cross-absorption and Western blotting, supported *R. helvetica* as the cause of disease. During 2003–2007, serologic findings in tickbite patients or in patients with fever of unknown origin from Switzerland, Italy, France, and Thailand were suggestive of acute or past *R. helvetica* infection (*5*,*36*). The few patients with a serology-based diagnosis had relatively mild, self-limited illnesses associated with headache and myalgias, and had a rash or eschar less frequently. Additional evaluation and isolation of the bacterium from clinical samples are needed to confirm the pathogenicity of *R. helvetica*.

We have detected in *I. ricinus* ticks a bacterium known as *R. monacensis* that was isolated from *I. ricinus* collected in 1998 in a park in Munich, Germany (*37*). This rickettsia is also found in the literature by other names such as the Cadiz agent found in Spain and *Rickettsia* IRS3 and IRS4, detected in Slovakia and Bulgaria. More recently, it has been identified in *I. ricinus* in Hungary (*38*). Recently, 2 human cases of infection with *R. monacensis* were documented in Spain (*39*). Investigators isolated this agent from the blood of 2 patients with Mediterranean spotted fever–like illnesses. The first patient was an 84-year-old man from La Rioja, Spain. He had fever and maculopapular rash without any inoculation eschar. The second patient was a 59-year-old woman from the Basque region of Spain. She had a history of a tickbite, fever, and a rash at the tickbite site (*39*). With our results, *R. monacensis* joins the list of autochthonous *Rickettsia* spp. confirmed as human pathogens in Morocco.

A total of 69% of *Haemaphysalis* spp. ticks tested harbored an incompletely described rickettsia. A closely related *glt*A sequence was found in GenBank as *Rickettsia endosymbiont of Haemaphysalis sulctata*. Duh et al. detected this bacterium in *Ha. sulcata* ticks collected from sheep and goats in southern Croatia (*40*). Using molecular analysis of the complete *glt*A gene and a portion of *omp*B, these authors detected this bacterium in 795 (22.8%) ticks tested. Similar to our findings, these researchers could not amplify DNA by PCR for the *omp*A gene with the primers Rr. 190.70-Rr. 190.701. Identification and isolation of this bacterium are needed until the name provisionally proposed by Duh et al, "*R. kastelanii*" (*40*), is accepted (*41*).

These findings demonstrate that species of ticks and several pathogens causing tick-transmitted diseases may

be prevalent in the same area. Our study also detected *R. slovaca*, *R. helvetica*, *R. monacensis*, *R. raoultii*, and an incompletely described rickettsia in Morocco. Clinicians in Morocco and those who may see patients returning from this country should be aware that many species of rickettsiae are present in this region and should consider a range of spotted fever rickettsial diseases in differential diagnosis of patients with febrile illnesses. Our data increase information on distribution of SFG rickettsiae in Morocco. Additional studies are needed to determine the epidemiologic and clinical relevance of different rickettsioses in this region.

Acknowledgment

We thank Christopher D. Paddock for editing the manuscript.

Dr Sarih is chief of the Laboratory of Medical Entomology at the Pasteur Institute in Casablanca, Morocco. His research interests include emerging tick-borne diseases.

References

1. Parola P, Raoult D. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin Infect Dis. 2001;32:897–928. DOI: 10.1086/319347
2. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the word: emerging diseases challenging old concepts. Clin Microbiol Rev. 2005;18:719–56. DOI: 10.1128/CMR.18.4.719-756.2005
3. Beati L, Meskini M, Thiers B, Raoult D. *Rickettsia aeschlimannii* sp. nov., a new spotted fever group rickettsia associated with *Hyalomma marginatum* ticks. Int J Syst Bacteriol. 1997;47:548–54.
4. Raoult D, Fournier PE, Abboud P, Caron F. First documented human *Rickettsia aeschlimannii* infection. Emerg Infect Dis. 2002;8:748–9.
5. Parola P, Miller RS, McDaniel P, Telford SR III, Rolain JM, Wongsrichanalai C, et al. Emerging rickettsioses of the Thai-Myanmar border. Emerg Infect Dis. 2003;9:592–5.
6. Beati L, Keirans JE. Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari: Ixodidae) based on mitochondrial 12S ribosomal DNA gene sequences and morphological characters. J Parasitol. 2001;87:32–48.
7. Brouqui P, Bacellar F, Baranton G, Birtles RJ, Bjoersdorff A, Blanco JR, et al. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. Clin Microbiol Infect. 2004;10:1108–32. DOI: 10.1111/j.1469-0691.2004.01019.x
8. Matsumoto K, Parola P, Brouqui P, Raoult D. *Rickettsia aeschlimannii* in *Hyalomma* ticks from Corsica. Eur J Clin Microbiol Infect Dis. 2004;23:732–4. DOI: 10.1007/s10096-004-1190-9
9. Loftis AD, Reeves WK, Szumlas DE, Abbassy MM, Helmy IM, Moriarity JR, et al. Rickettsial agents in Egyptian ticks collected from domestic animals. Exp Appl Acarol. 2006;40:67–81. DOI: 10.1007/s10493-006-9025-2
10. Pretorius AM, Birtles RJ. *Rickettsia aeschlimannii*: a new pathogenetic spotted fever group rickettsia, South Africa. Emerg Infect Dis. 2002;8:874.
11. Beati L, Raoult D. *Rickettsia massiliae* sp.nov., a new spotted fever group rickettsia. Int J Syst Bacteriol. 1993;43:839–40.
12. Cardenosa N, Segura F, Raoult D. Serosurvey among Mediterranean spotted fever patients of a new spotted fever group rickettsial strain (Bar29). Eur J Epidemiol. 2003;18:351–6. DOI: 10.1023/A:1023654400796
13. Matsumoto K, Ogawa M, Brouqui P, Raoult D, Parola P. Transmission of *Rickettsia massiliae* in the tick, *Rhipicephalus turanicus.* Med Vet Entomol. 2005;19:263–70. DOI: 10.1111/j.1365-2915.2005.00569.x
14. Eremeeva ME, Bosserman EA, Demma LJ, Zambrano ML, Blau DM, Dasch GA. Isolation and identification of *Rickettsia massiliae* from *Rhipicephalus sanguineus* ticks collected in Arizona. Appl Environ Microbiol. 2006;72:5569–77. DOI: 10.1128/AEM.00122-06
15. Vitale G, Mansueto S, Rolain JM, Raoult D. *Rickettsia massiliae* human isolation. Emerg Infect Dis. 2006;12:174–5.
16. Raoult D, Lakos A, Fenollar F, Beytout J, Brouqui P, Fournier PE. Spotless rickettsiosis caused by *Rickettsia slovaca* and associated with *Dermacentor* ticks. Clin Infect Dis. 2002;34:1331–6. DOI: 10.1086/340100
17. Rehacek J. *Rickettsia slovaca*, the organism and its ecology. Acta Scientiarum Naturalium Academiae Scientiarum Bohemoslovacae Brno. 1984;18:1–50.
18. Lakos A. Tick-borne lymphadenopathy — a new rickettsial disease? Lancet. 1997;350:1006. DOI: 10.1016/S0140-6736(05)64072-X
19. Raoult D, Berbis P, Roux V, Xu W, Maurin M. A new tick-transmitted disease due to *Rickettsia slovaca.* Lancet. 1997;350:112–3. DOI: 10.1016/S0140-6736(05)61814-4
20. Cazorla C, Enea M, Lucht F, Raoult D. First isolation of *Rickettsia slovaca* from a patient, France. Emerg Infect Dis. 2003;9:135.
21. Oteo JA, Ibarra V, Blanco JR, Martinez de Artola V, Marquez FJ, Portillo A, et al. *Dermacentor*-borne necrosis erythema and lymphadenopathy: clinical and epidemiological features of a new tick-borne disease. Clin Microbiol Infect. 2004;10:327–31. DOI: 10.1111/j.1198-743X.2004.00782.x
22. Gouriet F, Rolain JM, Raoult D. *Rickettsia slovaca* infection, France. Emerg Infect Dis. 2006;12:521–3.
23. Mediannikov OY, Matsumoto K, Samoylenko I, Drancourt M, Roux V, Rydkina E, et al. *Rickettsia raoultii* sp. nov., a new spotted fever group rickettsia associated with *Dermacentor* ticks in Europe and Russia. Int J Syst Evol Microbiol. 2008. In press.
24. Shpynov S, Fournier PE, Rudakov N, Tankibaev M, Tarasevich I, Raoult D. Detection of a rickettsia closely related to *Rickettsia aeschlimannii*, "*Rickettsia heilongjiangensis*", *Rickettsia* sp. strain RpA4, and *Ehrlichia muris* in ticks collected in Russia and Kazakhstan. J Clin Microbiol. 2004;42:2221–3. DOI: 10.1128/JCM.42.5.2221-2223.2004
25. Stanczak J. Detection of spotted fever group (SFG) rickettsiae in *Dermacentor reticulatus* (Acari: Ixodidae) in Poland. Int J Med Microbiol. 2006;296(Suppl 40):144–8. DOI: 10.1016/j.ijmm.2006.01.014
26. Dautel H, Dippel C, Oehme R, Hartelt K, Schettler E. Evidence for an increased geographical distribution of *Dermacentor reticulatus* in Germany and detection of *Rickettsia* sp. RpA4. Int J Med Microbiol. 2006;296(Suppl 40):149–56. DOI: 10.1016/j.ijmm.2006.01.013
27. Mediannikov OY, Parola P, Raoult D. Other tick-borne rickettsioses. In: Parola P, Raoult D, editors. Rickettsial diseases: infectious diseases and therapy. New York: Informa Healthcare; 2007. p. 139–62.
28. Estrada-Pena A, Bouattour A, Camicas J-L, Walker AR. Ticks of domestic animals in the Mediterranean region. Zaragosa (Spain): University of Zaragoza; 2004.
29. Parola P, Beati L, Cambon M, Raoult D. First isolation of *Rickettsia helvetica* from *Ixodes ricinus* ticks in France. Eur J Clin Microbiol Infect Dis. 1998;17:95–100.
30. Fournier PE, Fujita H, Takada N, Raoult D. Genetic identification of rickettsiae isolated from ticks in Japan. J Clin Microbiol. 2002;40:2176–81. DOI: 10.1128/JCM.40.6.2176-2181.2002
31. Nilsson K, Lindquist O, Pahlson C. Association of *Rickettsia helvetica* with chronic perimyocarditis in sudden cardiac death. Lancet. 1999;354:1169–73. DOI: 10.1016/S0140-6736(99)04093-3

32. Nilsson K, Pahlson C, Lukinius A, Eriksson L, Nilsson L, Lindquist O. Presence of *Rickettsia helvetica* in granulomatous tissue from patients with sarcoidosis. J Infect Dis. 2002;185:1128–38. DOI: 10.1086/339962
33. Nilsson K, Liu A, Pahlson C, Lindquist O. Demonstration of intracellular microorganisms (*Rickettsia* spp., *Chlamydia pneumoniae, Bartonella* spp. in pathological human aortic valves by PCR. J Infect. 2005;50:46–52. DOI: 10.1016/j.jinf.2003.10.009
34. Planck A, Eklund A, Grunewald J, Vene S. No serological evidence of *Rickettsia helvetica* infection in Scandinavian sarcoidosis patients. Eur Respir J. 2004;24:811–3. DOI: 10.1183/09031936.04.00014604
35. Fournier PE, Gunnenberger F, Jaulhac B, Gastinger G, Raoult D. Evidence of *Rickettsia helvetica* infection in humans, eastern France. Emerg Infect Dis. 2000;6:389–92.
36. Fournier PE, Allombert C, Supputamongkol Y, Caruso G, Brouqui P, Raoult D. Aneruptive fever associated with antibodies to *Rickettsia helvetica* in Europe and Thailand. J Clin Microbiol. 2004;42:816–8. DOI: 10.1128/JCM.42.2.816-818.2004
37. Simser JA, Palmer AT, Fingerle V, Wilske B, Kurtti TJ, Munderloh UG. *Rickettsia monacensis* sp. nov., a spotted fever group Rickettsia, from ticks (*Ixodes ricinus*) collected in a European city park. Appl Environ Microbiol. 2002;68:4559–66. DOI: 10.1128/AEM.68.9.4559-4566.2002
38. Sreter-Lancz Z, Szell Z, Kovacs G, Egyed L, Marialigeti K, Sreter T. Rickettsiae of the spotted-fever group in ixodid ticks from Hungary: identification of a new genotype ('Candidatus *Rickettsia kotlanii*'). Ann Trop Med Parasitol. 2006;100:229–36. DOI: 10.1179/136485906X91468
39. Jado I, Oteo JA, Aldamiz M, Gil H, Escudero R, Ibarra V, et al. *Rickettsia monacensis* and human disease, Spain. Emerg Infect Dis. 2007;13:1405–7.
40. Duh D, Punda-Polic V, Trilar T, Petrovec M, Bradaric N, Avsic-Zupanc T. Molecular identification of *Rickettsia felis*-like bacteria in *Haemaphysalis sulcata* ticks collected from domestic animals in southern Croatia. Ann N Y Acad Sci. 2006;1078:347–51. DOI: 10.1196/annals.1374.068
41. Raoult D, Fournier PE, Eremeeva M, Graves S, Kelly PJ, Oteo JA, et al. Naming of rickettsiae and rickettsial diseases. Ann N Y Acad Sci. 2005;1063:1–12. DOI: 10.1196/annals.1355.002

Address for correspondence: Philippe Parola, Unité des Rickettsies, Centre National de la Recherche Scientifique–Institut de Recherche pour le Développement, Unité Mixte de Recherche 6236, World Health Organization Collaborative Center for Rickettsioses and Other Arthropod Borne Bacterial Diseases, Faculté de Médecine, 27 Bd Jean Moulin, 13005 Marseille, France; email: philippe.parola@univmed.fr

Transmission of *Bartonella henselae* by *Ixodes ricinus*

Violaine Cotté,* Sarah Bonnet,* Danielle Le Rhun,* Evelyne Le Naour,* Alain Chauvin,† Henri-Jean Boulouis,‡ Benoit Lecuelle,‡ Thomas Lilin,‡ and Muriel Vayssier-Taussat*

Bartonella spp. are facultative intracellular bacteria associated with several emerging diseases in humans and animals. *B. henselae* causes cat-scratch disease and is increasingly associated with several other syndromes, particularly ocular infections and endocarditis. Cats are the main reservoir for *B. henselae* and the bacteria are transmitted to cats by cat fleas. However, new potential vectors are suspected of transmitting *B. henselae*, in particular, *Ixodes ricinus*, the most abundant ixodid tick that bites humans in western Europe. We used a membrane-feeding technique to infect *I. ricinus* with *B. henselae* and demonstrate transmission of *B. henselae* within *I. ricinus* across developmental stages, migration or multiplication of *B. henselae* in salivary glands after a second meal, and transmission of viable and infective *B. henselae* from ticks to blood. These results provide evidence that *I. ricinus* is a competent vector for *B. henselae*.

Bartonella spp. are facultative intracellular bacteria associated with several emerging diseases in humans and animals (*1*). Domestic animals and wildlife represent a large reservoir for *Bartonella* spp., and at least 10 species or subspecies have been reported to cause zoonotic infections. *B. henselae* causes cat-scratch disease, possibly the most common zoonosis acquired from domestic animals in industrialized countries and is becoming increasingly associated with other syndromes, particularly ocular infections and endocarditis (*2–6*). Although cat fleas are well-established vectors for *B. henselae* (*7–10*), transmission by other arthropods, in particular ticks, has been suggested (*11–13*). *Ixodes ricinus* is the most widespread and abundant ixodid tick in western Europe and is frequently associated with bites in humans. It is a vector of emerging zoonotic pathogens including *Borrelia burgdorferi* sensu lato (*14*), *Anaplasma phagocytophilum* (*15*), and *Babesia* spp. (*16*).

Direct proof of transmission of *Bartonella* spp. by a tick was reported by Noguchi in 1926 (*17*), who described experimental transmission of *B. bacilliformis* (cause of Oroya fever) to monkeys by *Dermacentor andersoni*. In this study, ticks were allowed to feed on infected monkeys for 5 days. After removal, partially engorged ticks were placed on healthy monkeys in which disease then developed. This study showed that ticks could acquire and transmit the bacteria but did not demonstrate their vector competence or transtadial transmission throughout the tick's life cycle. Since this early study, the role of ticks in *Bartonella* spp. transmission has been strongly implied but never definitively demonstrated. *Bartonella* spp. DNA was detected in questing and engorged nymphs and adults *Ixodes* spp. collected in North America, Europe, and Asia (*13*,*18–26*). If one considers that ixodid ticks feed only once per stage, *Bartonella* spp. DNA in questing ticks suggests transtadial transmission of these bacteria.

Other observations support *Bartonella* spp. transmission by ticks. Co-occurrence of *Bartonella* spp. with known tick-borne pathogens such as *B. burgdorferi* sensu lato, *A. phagocytophilum*, or *Babesia* spp. is not a rare event in ticks and hosts (*13*,*19*,*24*,*27*). A study conducted in a veterinary hospital in the United States (California) demonstrated that all dogs with endocarditis and infected with *Bartonella* spp. were also seropositive for *A. phagocytophilum* (*28*). In humans, several case studies have reported patients with concurrent *Bartonella* seropositivity and detection of *Bartonella* spp. DNA in their blood, along with *B. burgdorferi* infection of the central nervous system after tick bites (*11*,*29*). Moreover, *Bartonella* spp.

*Institut National de la Recherche Agronomique, Maisons-Alfort, France; †École Nationale Vétérinaire de Nantes, Nantes, France; and ‡École Nationale Vétérinaire d'Alfort, Maisons-Alfort

DOI: 10.3201/eid1407.071110

DNA has been detected in human blood cells after a tick bite (*30*), and 3 patients with *B. henselae* bacteremia who had no history of contact with cats but had sustained tick bites were reported in Texas (*12*). Finally, tick exposure was determined to be a risk factor associated with *B. vinsonii* seropositivity in dogs (*31*).

Because *Bartonella* spp. are emerging human pathogens and *Ixodes* spp. can transmit a large spectrum of pathogens to humans, the capability of *Ixodes* spp. in transmitting human pathogenic *Bartonella* spp. should be determined. We used a membrane-feeding technique to infect *I. ricinus* with *B. henselae*, and investigated transtadial and transovarial transmission of viable and infective bacteria and putative transmission from tick saliva to blood during artificial blood meals.

Materials and Methods

Ticks

I. ricinus ticks were collected from the forest of Gâvre (Loire-Atlantique, France) in 2006 by flagging vegetation as described (*32*). Ticks were reared and maintained in chambers with a relative humidity of 80%–90% at 22°C before feeding on artificial skin. A total of 217 whole ticks were tested for *Bartonella* DNA.

Culturing of *B. henselae*

B. henselae (Houston-1 ATCC 49 882) were grown on 5% defibrinated sheep blood Columbia agar (CBA) plates, which were incubated at 35°C in an atmosphere of 5% CO_2. After 10 days, bacteria were harvested, suspended in sterile phosphate-buffered saline (PBS), and used immediately for artificial feeding of ticks.

Housing of Cats

Three healthy male European cats were used (12–14 months of age, weight = 3.2–4.6 kg at the beginning of the study). These cats, which were bred by Harlan (Indianapolis, IN, USA), were imported at 9 months of age. Before the study, animals underwent clinical examinations and showed no signs of disease. Absence of *Bartonella* spp. was confirmed by blood culture, tests for DNA of *Bartonella* spp., and serologic analysis. Animals were housed singly in cages in compliance with European guidelines (cage surface 6,000 cm^2, cage height 50 cm). Animals were allowed to acclimate for 3 months to the facility, diet, and handling before the first blood sample was taken and subsequent infection. Animals were fed ad libitum with standard feline maintenance diet (Harlan) and received water with no restrictions. Animal care was provided in accordance with the good animal care practice.

Feeding of *I. ricinus* Ticks with Ovine Blood

The general experimental framework of artificial feeding is shown in Figure 1. Ovine blood used in all experiments was obtained from 3 sheep reared at the National Veterinary School in Nantes, France. Absence of *Bartonella* spp. in the blood of these sheep was confirmed by culture assay and tests for DNA of *Bartonella* spp. by PCR. Lithium heparin–coated vacutainer tubes (Becton Dickinson, Le Pont de Claix-Cedex, France) were used to draw blood by venipuncture. Blood was depleted of functional complement by heat treatment (incubation for 30 min at 56°C) before use. To avoid fungal and bacterial contamination during feeding experiments, decomplemented blood was supplemented with fosfomycin (20 μg/mL) and amphotericin B (0.25 μg/mL), which were previously determined to have no effect on *B. henselae* viability.

Feeding of Ticks with *B. henselae*–Infected Blood

The method of artificial feeding used in this study was adapted from the method of Bonnet et al. (*33*). Ticks were placed in 75-cm^2 tissue culture flasks pierced at the top to accommodate a 4-cm diameter glass feeder. The feeder apparatus was closed with a parafilm membrane at the top and

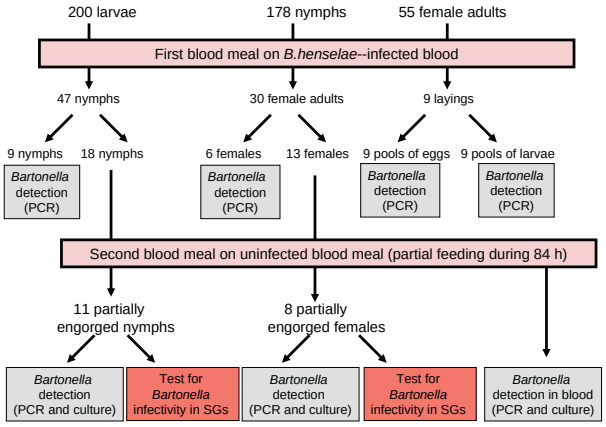


Figure 1. Experimental framework of *Ixodes ricinus* tick infection by *Bartonella henselae*–infected blood. Ticks (200 larvae, 178 nymphs, and 55 female adults) were engorged by feeding through artificial skin on *B. henselae*–infected blood for 5 days for larvae, 12 days for nymphs, and 21 days for adults. Larvae and nymphs were allowed to molt and engorged females were allowed to lay eggs. To evaluate transtadial and transovarial transmission, *Bartonella* spp. DNA was detected by PCR in salivary glands (SGs) and carcasses of 9 nymphs, 6 female adults, 9 pools of eggs, and resulting pools of larvae. Eighteen nymphs and 13 adult females fed on infected blood at preceding stages were refed for 84 h on noninfected blood. *Bartonella* spp. DNA was detected by PCR in SGs of 7 engorged nymphs and 3 engorged female adults. *B. henselae* colonies were isolated from SGs of 3 nymphs and 4 adults and from blood removed from feeders. Infectivity of *B. henselae* in SGs was tested by infecting 2 cats with 1 pair of SGs from a potentially infected nymph and 1 pair of SGs from a potentially infected adult, respectively.

a gerbil or rabbit skin membrane at the bottom. Gerbil skins were used for feeding larvae and rabbit skins for feeding nymphs and adults (*33*). To attract ticks, a constant temperature (37°C) was maintained by a water-jacket circulation system through the glass feeder. For blood infection, 10 μL of *B. henselae* suspension at a concentration of 10^9 CFU/mL in PBS was added to 10 mL of blood supplemented with fosfomycin and amphotericin B. The culture box containing ticks was placed under the feeding apparatus, and 3 mL of *B. henselae*–infected ovine blood, changed twice a day, was introduced until the ticks were replete. To feed adult ticks, an equal number of males (for reproduction) and females were used. Separate apparatuses were used to engorge 200 larvae, 178 nymphs, and 55 female adults. As a control, 50 nymphs were fed under the same conditions on uninfected decomplemented ovine blood.

After feeding, larvae and nymphs were allowed to molt to nymphs and adults, respectively, and engorged females were allowed to lay eggs. Between each feeding on skin, ticks were starved for at least 2 months.

Feeding with Uninfected Blood of Ticks Infected with *B. henselae* at Preceding Stages

Nymphs and adults fed on blood infected with *B. henselae* at preceding stages were fed with uninfected, decomplemented, ovine blood in 2 glass feeders as described above. After 84 h of refeeding, nymphs and females attached to the skin were removed and dissected. *Bartonella* spp. DNA was detected every 24 h in blood from the first 48 h of attachment onward. At each time point, 3 mL of blood was removed and centrifuged for 30 min at 3,000 × *g*. The supernatant was aspirated, and the pellet (200 μL) was used to detect bacterial DNA. After 84 h of feeding, 10 μL of blood was used for *B. henselae* culture.

Tick Dissection

Salivary glands (SGs) from *I. ricinus* adults and nymphs were dissected under a magnifying glass in sterile PBS. All dissection material was cleaned with DNA-off (Eurobio, Courtaboeuf, France) and rinsed with sterile water between each sample. Individual pairs of tick SGs and the remaining tick carcasses were suspended in 150 μL of PBS before culture and used to infect cats or frozen at –80°C until DNA extraction.

Detection of *B. henselae* in Tick and Blood Samples

DNA Extraction

Carcasses (ticks without SGs), entire ticks, and pools of 50 larvae were mechanically disrupted as described (*34*). Eggs were crushed by using a microtissue grinder and dissected SGs were directly used for DNA extraction. DNA was extracted from all tick samples by using the Nucleospin Tissue kit according to the manufacturer's instructions (Macherey-Nagel, Duren, Germany). DNA was extracted from blood samples by using the Nucleospin Blood Quick Pure kit (Macherey-Nagel). For carcasses and entire ticks, the final elution volume was 100 μL for adults and 30 μL for nymphs. For DNA from SGs, the final elution volume was 50 μL for adults and 20 μL for nymphs. Pools of eggs, larvae, and blood DNA were eluted in volumes of 50 μL.

PCR Amplification

Efficiency of tick DNA extraction was evaluated in all samples by amplification of a fragment of the tick mitochondrial 16S rRNA gene by using tick-specific primers TQ16S+1F (5′-CTGCTCA ATGATTTTTTAAATTGCTGTGG-3′) and TQ16S-2R (5′-ACGCTGTTATCCCTAGAG-3′) as described (*35*). A seminested PCR was used to detect *B. henselae* DNA. Amplification was initially performed with 5 μL of DNA extract and universal bacteria primers amplifying a 535-bp fragment of the 16S rRNA gene: pc535 (5′-GTATTACCGCGGCTGCTGGCA-3′) and p8 (5′AGAG TTTGATCCTGGCTCAG-3′).

The second seminested amplification was performed with 5 μL of a 100-fold dilution of the first PCR product and primers pc535 and bsp16F (5′-TCTCTACGGAATAACACAGA-3′) a 16S rRNA *Bartonella* spp.–specific primer, and resulted in amplification of a 337-bp product. The PCR cycle was identical for both amplification reactions: an initial denaturation step for 8 min at 94°C; 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 54°C, and extension for 1 min at 72°C; and a final extension step at 72°C for 10 min. Each reaction was conducted in a total volume of 25 μL with 0.5 μmol/μL of each primer, 2.5 mmol/L of each dNTP, 2.5 μL of 10× PCR buffer, and 1 U of *Taq* DNA polymerase (Takara Biomedical Group, Shiga, Japan). Negative (ticks fed on uninfected ovine blood) and positive (*B. bacilliformis* DNA to easily detect any cross-contamination) controls were included in each assay. All PCRs were performed in a thermocycler MyCycler (Bio-Rad, Strasbourg, France).

Sequencing and Sequence Analysis

The expected 337-bp PCR product was isolated by agarose gel electrophoresis, excised from the gel, purified by using NucleoSpin Extract II (Macherey-Nagel), and sent to QIAGEN (Hilden, Germany) for direct sequencing. Sequences were compared with known sequences listed in the GenBank nucleotide sequence databases by using the BLAST search option of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

B. henselae Culture from Tick SGs and Blood

After refeeding and dissection, 4 pairs of SGs from 4

potentially infected adults females and 3 nymphs were incubated in 1 mL of Schneider *Drosophila* medium (Invitrogen, Cergy-Pontoise, France) at 35°C in an atmosphere of 5% CO_2. After 6 days of incubation, 10-μL samples were placed on CBA plates. Ten microliters of blood was removed from the glass feeder after 84 h of refeeding with adults suspected of having *B. henselae* and incubated for 6 days in Schneider *Drosophila* medium before being placed on CBA plates.

Infection of Cats with Adult and Nymph SGs Potentially Infected with *B. henselae*

One cat was intravenously infected with 150 μL of PBS containing SGs from refed adult ticks suspected of having *B. henselae*; another cat was infected with 150 μL of PBS. Before handling, cats were anesthetized with ketamine (10–15 mg/kg bodyweight) and diazepam (100 μg/kg bodyweight) given intravenously. One milliliter of blood was obtained from the jugular vein at days 7, 14, and 21 postinfection, and 100 μL of blood dilutions (1:10, 1:100, and 1:1,000) was directly placed on sheep blood CBA plates. Colonies were counted and CFU/mL was estimated after incubation for 10 days at 35°C in an atmosphere of 5% CO_2.

Results

Detection of *Bartonella* spp. DNA in Questing *I. ricinus* Ticks

We tested 217 ticks collected in the forest of Gavre to determine prevalence of *Bartonella* spp. DNA. None of the 98 nymphs, 49 female ticks, and 70 male ticks collected in the forest showed amplification of the *Bartonella* spp. 16S rRNA gene.

Transstadial Transmission of *B. henselae* DNA by *I. ricinus* Ticks

A total of 433 *I. ricinus* ticks were used in 2 independent experiments with feeding on artificial skin. Of these ticks, 169 (84.5%) of 200 larvae, 111 (62.3%) of 178 nymphs, and 19 (34.5%) of 55 female adults were successfully engorged and spontaneously detached. After feeding and detachment, all ticks were maintained in humidity chambers to enable molting or laying of eggs. After 3 months, 47 larvae (27.8%) and 58 nymphs (30.1%) molted into nymphs and adults (28 males and 30 females), respectively. Of the 19 engorged females, 9 laid eggs.

Nine nymphs exposed to infection as larvae were tested for *B. henselae* DNA. All carcasses showed amplification of the expected 337-bp DNA fragment, and no amplification product was obtained from SGs. Four carcasses of 6 females exposed to *B. henselae* as nymphs showed the 337-bp DNA fragment, and no amplification product was obtained with SGs (Table, Figure 2). No amplified fragment was detected with DNA extracts from SGs or carcasses of control ticks fed on uninfected blood. All PCR products were sequenced and shared 100% identity with the 16S rRNA gene of *B. henselae* Houston-1 (Genbank accession no. BX897699).

Transovarial Transmission of *B. henselae* DNA by *I. ricinus* Ticks

Among 9 pools of eggs laid by females fed on *B. henselae*–infected blood, 3 showed amplification of the expected *Bartonella* spp.–specific 337-bp DNA fragment. No amplification was obtained with larvae from eggs positive or negative for *Bartonella* spp. DNA (Figure 2).

Transmission of Viable and Infective *B. henselae* by *I. ricinus* Ticks

Eighteen nymphs and 13 female adults fed on *B. henselae*–infected blood at preceding life stages were refed for 84 h with uninfected blood. DNA extracts were prepared from SGs and carcasses of 7 partially engorged nymphs and 3 partially engorged females. *Bartonella* spp. DNA was amplified from carcasses and SGs of 4 nymphs and 1 female. For 1 nymph and 1 female, the specific 337-bp DNA fragment was amplified only in SG DNA extracts but not in carcass extracts (Table; Figure 3, panel A). Four pairs of SGs from 4 partially engorged females and 3 pairs of SGs from 3 partially engorged nymphs were incubated separately in Schneider *Drosphila* medium for 6 days before being placed on sheep blood agar. *B. henselae* colonies appeared after 7 days for all SGs tested, which indicated the presence of viable bacteria in SGs from adults and nymphs.

To determine whether *B. henselae* in SGs of nymphs and adults were infective, we infected 2 healthy cats with 1 pair of SGs from a nymph and 1 pair of SGs from a female adult. The cat infected with SGs from an adult became bacteremic 7 days after infection (5×10^5 CFU/mL at day7, 6×10^6 CFU/mL at day 14, and 1×10^6 CFU/mL at day 21), and the cat infected with SGs from a nymph became bacte-

Table. Transtadial transmission of *Bartonella* spp. DNA in *Ixodes ricinus* ticks after feeding through artificial skin on *B. henselae*–infected ovine blood

	PCR detection after infectious blood meal and molting, no. PCR-positive samples/no. samples tested (%)		PCR detection after partial refeeding, no. PCR-positive samples/no. samples tested (%)	
I. ricinus stage	Carcasses	Salivary glands	Carcasses	Salivary glands
Nymph	9/9 (100)	0/9 (0)	4/7 (57)	5/7 (71)
Female adult	4/6 (67)	0/6 (0)	1/3 (33)	2/3 (67)

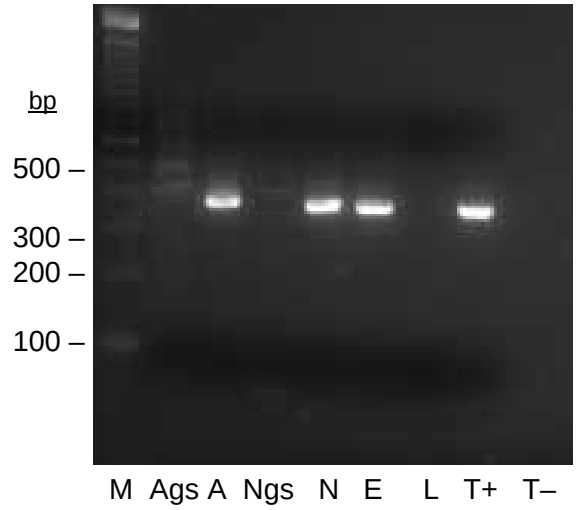


Figure 2. Seminested PCR detection of *Bartonella* spp. DNA in *Ixodes ricinus* ticks fed on *B. henselae*–infected ovine blood at preceding stage. Lane M, 100-bp DNA molecular mass marker; lane Ags, salivary glands of a female adult fed on infected blood as a nymph; lane A, carcass of a female adult fed on infected blood as a nymph; lane Ngs, salivary glands of a nymph fed on infected blood as a larva; lane N, carcass of a nymph fed on infected blood as a larva; lane E, eggs laid by female adult fed on infected blood; lane L, larvae hatched from female adult fed on infected blood; lane T+, *B. bacilliformis* DNA; lane T–, nymph fed on uninfected ovine blood.

remic after 14 days of infection (8×10^5 CFU/mL at day 14 and 2×10^7 CFU/mL at day 21).

Blood samples from feeders were obtained every 12 h for the first 48 h to detect *B. henselae*. In the first 60 h, *B. henselae* DNA or viable *B. henselae* was not detected in blood samples. After 72 h of refeeding of infected ticks, *Bartonella* spp. DNA was successfully amplified (Figure 3, panel B), and *B. henselae* colonies were isolated from these blood samples after preincubation for 6 days in Schneider *Drosophila* medium, which indicated viability of the transmitted bacteria.

Discussion

This study demonstrated transmission of *B. henselae* by *I. ricinus* ticks across different developmental stages, migration and multiplication of viable and infective *B. henselae* in SGs after a second blood meal, and transmission of *B. henselae* from ticks to blood. These findings indicate that *I. ricinus* is a competent vector for *B. henselae*.

Vector biologists and epidemiologists have suggested that ticks may play a role in transmission of *Bartonella* spp. (*11*,*12*,*19*,*23*,*25*,*28*,*29*). This suggestion was based on indirect data for detection of bacterial DNA in ticks (*18*,*19*,*24*), humans exposed to tick bites (*30*), or serologic evidence of co-infection of humans with pathogens known to be transmitted by ticks (*11*,*36*). Difficulties in rearing *I. ricinus* and lack of a rodent model for *B. henselae* infection may explain the absence of data demonstrating the role of this tick as a vector of *B. henselae*. Recent development of an artificial method suitable for feeding ticks (*33*) enabled us to study experimental infection of ticks with blood containing *B. henselae*, to monitor *B. henselae* through various tick stages, and to evaluate putative transmission of bacteria from the tick to blood.

To select a tick population with the lowest *Bartonella* spp. DNA prevalence, we estimated the prevalence of *Bartonella* spp. DNA in questing *I. ricinus* collected in different areas in France. The lowest *Bartonella* spp. DNA prevalence was in Loire-Atlantique (*19* and unpub. data). We thus used ticks collected in this area for our study.

We detected *B. henselae* DNA in 100% of carcasses from nymphs and 67% of carcasses from adults fed on ovine blood containing *B. henselae* at their preceding stages. No *B. henselae* DNA was amplified in corresponding SGs in a nested PCR, which is more sensitive than amplification of the classic citrate synthase gene. This result demonstrated that bacteria could be ingested by *I. ricinus* larvae and nymphs during feeding on artificial skin and that bacterial DNA was maintained in the tick after molting. However, no or undetectable numbers reached the SGs.

Although bacterial DNA was detected in eggs laid by females fed on blood containing *B. henselae*, larvae obtained from these eggs were PCR-negative for *B. henselae*. This finding suggests external contamination of eggs with DNA rather than transovarial persistence of bacteria.

When molted nymphs and female ticks potentially contaminated with *B. henselae* at their previous developmental stage were refed on uninfected blood, viable *B. henselae* were detected in SGs after 84 h of engorgement. Two hypotheses could explain the absence of detectable bacterial DNA in SGs after an infected blood meal and molting, when it becomes detectable after a partial refeeding blood meal. The first hypothesis is that the 84-h refeeding period may act as a stimulus and enable migration of bacteria from the gut to SGs of the tick as previously described for *B. burgdorferi* sensu lato (*14*). The second hypothesis is that this refeeding period may stimulate multiplication of bacteria already present in SGs, but at undetectable levels. More investigations are needed to validate one of these hypotheses. Bacteria located in SGs of nymphs and adults are infective because injection of 1 pair of infected SGs into cats induced high levels of bacteremia. Cats became bacteremic in the first 2 weeks after injection, as described for cat infection with *B. henselae* by fleas, and bacteremia levels were similar to those observed in cats infected by flea bites (*7*,*8*).

Viable *B. henselae* in blood after 72 h of feeding of ticks with *B. henselae*–infected ticks demonstrated its

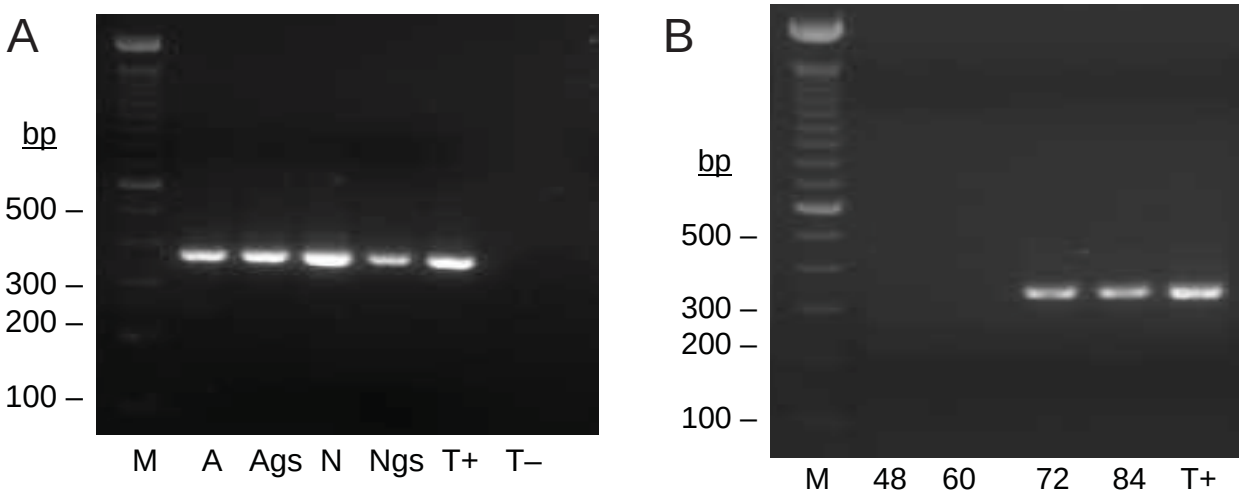


Figure 3. Seminested PCR detection of *Bartonella* spp. DNA after partial refeeding of infected ticks. A) *Bartonella* spp. DNA detection in *Ixodes ricinus* ticks fed on *B. henselae*–infected blood at previous development stages and refed for 84 h on uninfected blood. Lane M, 100-bp DNA molecular mass; lane A, carcass of female adult; lane Ags, salivary glands of female adult, lane N, carcass of nymph; lane Ngs, salivary glands of nymph; lane T+, *B. bacilliformis* DNA; laneT–, nymph fed on uninfected ovine blood. B) *Bartonella* spp. DNA detection in blood isolated from feeders. Lane M, 100-bp DNA molecular mass marker; lane 48, ovine blood after 48 h of tick attachment on skin; lane 60, ovine blood after 60 h of tick attachment on skin; lane 72, ovine blood after 72 h of tick attachment on skin; lane 84, ovine blood after 84 h of tick attachment on skin; lane T+, *B. bacilliformis* DNA.

transmission from the tick to the blood by mouthparts of the ticks. The duration of multiplication or migration described above would explain such a delay in bacterial transmission.

Because our results have demonstrated competence of *I. ricinus* for transmission of *B. henselae*, cat models of *B. henselae* transmission by ticks are needed to confirm that cats can be infected with *B. henselae* by tick bites. Further investigations are also needed to evaluate the capacity of *I. ricinus* to transmit *B. henselae* to cats and humans. Such transmission could occur because cats, although not common hosts for *I. ricinus*, can be infested with this tick. In France, attached *I. ricinus* are commonly found on cats brought to veterinarians (J. Guillot, pers. comm.). In Great Britain, Ogden et al. (*37*) reported cats with woodland and moorland habitats as hosts for *I. ricinus*. Podsiadly et al. (*38*) reported *B. henselae* in cats and in *I. ricinus* removed from those cats in Poland.

In conclusion, we demonstrated by using feeding on artificial skin that *B. henselae*, the cause of cat-scratch disease in humans, could be transmitted by ticks through saliva. Although further investigations are needed to clarify the epidemiology of such transmission, health authorities must take into account the possibility of bartonellosis in persons exposed to tick bites, and *B. henselae* must be identified as a tick-borne pathogen.

Acknowledgments

We thank A. Agoulon and J.P. Delatre for collecting ticks.

This study was supported by Merial Laboratory, Lyon, France, and the French National Institute for Agronomic Science, Paris, France.

Ms Cotté is a doctoral student in microbiology at the Unité Mixte de Recherche 956 in Maisons-Alfort, France. Her research interest is transmission of *Bartonella* spp. by ticks.

References

1. Boulouis HJ, Chang CC, Henn JB, Kasten RW, Chomel BB. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. Vet Res. 2005;36:383–410. DOI: 10.1051/vetres:2005009
2. Anderson BE, Neuman MA. *Bartonella* spp. as emerging human pathogens. Clin Microbiol Rev. 1997;10:203–19.
3. Oteo JA, Castilla A, Arosey A, Blanco JR, Ibarra V, Morano LE. Endocarditis due to *Bartonella* spp. Three new clinical cases and Spanish literature review [in Spanish]. Enferm Infecc Microbiol Clin. 2006;24:297–301. DOI: 10.1157/13089663
4. Marsilia GM, La Mura A, Galdiero R, Galdiero E, Aloj G, Ragozzino A. Isolated hepatic involvement of cat scratch disease in immunocompetent adults: enhanced magnetic resonance imaging, pathological findings, and molecular analysis–two cases. Int J Surg Pathol. 2006;14:349–54. DOI: 10.1177/1066896906291780
5. Curi AL, Machado DO, Heringer G, Campos WR, Orefice F. Ocular manifestation of cat-scratch disease in HIV-positive patients. Am J Ophthalmol. 2006;141:400–1. DOI: 10.1016/j.ajo.2005.08.072
6. Chomel BB, Boulouis HJ, Maruyama S, Breitschwerdt EB. *Bartonella* spp. in pets and effect on human health. Emerg Infect Dis. 2006;12:389–94.
7. Chomel BB, Kasten RW, Floyd-Hawkins K, Chi B, Yamamoto K, Roberts-Wilson J, et al. Experimental transmission of *Bartonella henselae* by the cat flea. J Clin Microbiol. 1996;34:1952–6.
8. Higgins JA, Radulovic S, Jaworski DC, Azad AF. Acquisition of the cat scratch disease agent *Bartonella henselae* by cat fleas (Siphonaptera:Pulicidae). J Med Entomol. 1996;33:490–5.

9. Foil L, Andress E, Freeland RL, Roy AF, Rutledge R, Triche PC, et al. Experimental infection of domestic cats with *Bartonella henselae* by inoculation of *Ctenocephalides felis* (Siphonaptera:Pulicidae). J Med Entomol. 1998;35:625–8.
10. Finkelstein JL, Brown TP, O'Reilly KL, Wedincamp J Jr, Foil LD. Studies on the growth of *Bartonella henselae* in the cat flea (Siphonaptera:Pulicidae). J Med Entomol. 2002;39:915–9.
11. Eskow E, Rao RV, Mordechai E. Concurrent infection of the central nervous system by *Borrelia burgdorferi* and *Bartonella henselae*: evidence for a novel tick-borne disease complex. Arch Neurol. 2001;58:1357–63. DOI: 10.1001/archneur.58.9.1357
12. Lucey D, Dolan MJ, Moss CW, Garcia M, Hollis DG, Wegner S, et al. Relapsing illness due to *Rochalimaea henselae* in immunocompetent hosts: implication for therapy and new epidemiological associations. Clin Infect Dis. 1992;14:683–8.
13. Holden K, Boothby JT, Kasten RW, Chomel BB. Co-detection of *Bartonella henselae, Borrelia burgdorferi*, and *Anaplasma phagocytophilum* in *Ixodes pacificus* ticks from California, USA. Vector Borne Zoonotic Dis. 2006;6:99–102. DOI: 10.1089/vbz.2006.6.99
14. De Silva AM, Fikrig E. Growth and migration of *Borrelia burgdorferi* in *Ixodes ricinus* ticks during blood feeding. Am J Trop Med Hyg. 1995;53:397–404.
15. Raoult D, Roux V. Rickettsioses as paradigms of new or emerging infectious diseases. Clin Microbiol Rev. 1997;10:694–719.
16. Gorenflot A, Moubri K, Precigout E, Carcy B, Schetters TP. Human babesiosis. Ann Trop Med Parasitol. 1998;92:489–501. DOI: 10.1080/00034989859465
17. Noguchi H. Etiology of Oroya fever: V. The experimental transmission of *Bartonella bacilliformis* by ticks (*Dermacentor andersoni*). J Exp Med. 1926;44:729–34. DOI: 10.1084/jem.44.5.729
18. 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. DOI: 10.1128/JCM.39.4.1221-1226.2001
19. Halos L, Jamal T, Maillard R, Beugnet F, Le Menach A, Boulouis HJ, et al. Evidence of *Bartonella* sp. in questing adult and nymphal *Ixodes ricinus* ticks from France and co-infection with *Borrelia burgdorferi* sensu lato and *Babesia* sp. Vet Res. 2005;36:79–87. DOI: 10.1051/vetres:2004052
20. Morozova OV, Cabello FC, Dobrotvorsky AK. Semi-nested PCR detection of *Bartonella henselae* in *Ixodes persulcatus* ticks from Western Siberia, Russia. Vector Borne Zoonotic Dis. 2004;4:306–9. DOI: 10.1089/vbz.2004.4.306
21. Kim CM, Kim JY, Yi YH, Lee MJ, Cho MR, Shah DH, et al. Detection of *Bartonella* species from ticks, mites and small mammals in Korea. J Vet Sci. 2005;6:327–34.
22. Schouls LM, van de Pol I, Rijpekema SG, Schot CS. Detection and identification of *Ehrlichia, Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J Clin Microbiol. 1999;37:2215–22.
23. Sanogo YO, Zeaiter Z, Caruso G, Merola F, Shpynov S, Brouqui P, et al. *Bartonella henselae* in *Ixodes ricinus* ticks (Acari: Ixodida) removed from humans, Belluno province, Italy. Emerg Infect Dis. 2003;9:329–32.
24. Adelson ME, Rao RV, Tilton RC, Cabets K, Eskow E, Fein L, et al. Prevalence of *Borrelia burgdorferi, Bartonella* spp., *Babesia microti*, and *Anaplasma phagocytophila* in *Ixodes scapularis* ticks collected in northern New Jersey. J Clin Microbiol. 2004;42:2799–801. DOI: 10.1128/JCM.42.6.2799-2801.2004
25. Podsiadly E, Chmielewski T, Sochon E, Tylewska-Wierzbanowska S. *Bartonella henselae* in *Ixodes ricinus* ticks removed from dogs. Vector Borne Zoonotic Dis. 2007;7:189–92. DOI: 10.1089/vbz.2006.0587
26. Rar VA, Fomenko NV, Dobrotvorsky AK, Livanova NN, Rudakova SA, Fedorov EG, et al. Tickborne pathogen detection, Western Siberia, Russia. Emerg Infect Dis. 2005;11:1708–15.
27. Solano-Gallego L, Bradley J, Hegarty B, Sigmon B, Breitschwerdt E. *Bartonella henselae* IgG antibodies are prevalent in dogs from southeastern USA. Vet Res. 2004;35:585–95. DOI: 10.1051/vetres:2004034
28. 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. DOI: 10.1892/0891-6640(2004)18<56:APSOCI>2.0.CO;2
29. Podsiadly E, Chmielewski T, Tylewska-Wierzbanowska S. *Bartonella henselae* and *Borrelia burgdorferi* infections of the central nervous system. Ann N Y Acad Sci. 2003;990:404–6.
30. Morozova OV, Chernousova NI, Morozov IV. Detection of the *Bartonella* DNA by the method of nested PCR in patients after tick bites in Novosibirsk region [in Russian]. Mol Gen Mikrobiol Virusol. 2005;4:14–7.
31. Pappalardo BL, Correa MT, York CC, Peat CY, Breitschwerdt EB. Epidemiologic evaluation of the risk factors associated with exposure and seroreactivity to *Bartonella vinsonii* in dogs. Am J Vet Res. 1997;58:467–71.
32. Vassalo M, Paul RE, Pereiz-Eid C. Temporal distribution of the annual nymphal stock of *Ixodes ricinus* ticks. Exp Appl Acarol. 2000;24:941–9. DOI: 10.1023/A:1010669003887
33. Bonnet S, Jouglin M, Malandrin L, Becker C, Agoulon A, L'Hostis M, et al. Transstadial and transovarial persistence of *Babesia divergens* DNA in *Ixodes ricinus* ticks fed on infected blood in a new skin-feeding technique. Parasitology. 2006;134:197–207. DOI: 10.1017/S0031182006001545
34. Halos L, Jamal T, Vial L, Maillard R, Suau A, Le Menach A, et al. Determination of an efficient and reliable method for DNA extraction from ticks. Vet Res. 2004;35:709–13. DOI: 10.1051/vetres:2004038
35. Black WC IV, Piesman J. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc Natl Acad Sci U S A. 1994;91:10034–8. DOI: 10.1073/pnas.91.21.10034
36. Kordick SK, Breitschwerdt EB, Hegarty BC, Southwick KL, Colitz CM, Hancock SI. Coinfection with multiple tick-borne pathogens in a Walker Hound kennel in North Carolina. J Clin Microbiol. 1999;37:2631–8.
37. Ogden NH, Cripps P, Davison CC, Owen G, Parry JM, Timms BJ, et al. The ixodid tick species attaching to domestic dogs and cats in Great Britain and Ireland. Med Vet Entomol. 2000;14:332–8. DOI: 10.1046/j.1365-2915.2000.00244.x
38. Podsiadly E, Chmielewski T, Marczak R, Sochon E, Tylewska-Wierzbanowska S. *Bartonella henselae* in the human environment in Poland. Scand J Infect Dis. 2007;39:956–62. DOI: 10.1080/00365540701449385

Address for correspondence: Muriel Vayssier-Taussat, Unité Mixte de Recherche, Biologique et Immunologie Moléculaires des Infections Parasitaires et Fongiques, Agence Francaise de Securite Sanitaire des Aliments, 23 Rue du General de Gaulle, Maisons-Alfort 94700, France; email: mvayssier@vet-alfort.fr

Seasonality, Annual Trends, and Characteristics of Dengue among Ill Returned Travelers, 1997–2006

Eli Schwartz,*† Leisa H. Weld,‡ Annelies Wilder-Smith,§ Frank von Sonnenburg,¶ Jay S. Keystone,# Kevin C. Kain,#** Joseph Torresi,††‡‡ and David O. Freedman,§§ for the GeoSentinel Surveillance Network[1]**

We examined seasonality and annual trends for dengue cases among 522 returned travelers reported to the international GeoSentinel Surveillance Network. Dengue cases showed region-specific peaks for Southeast Asia (June, September), South Central Asia (October), South America (March), and the Caribbean (August, October). Travel-related dengue exhibited annual oscillations with several epidemics occurring during the study period. In Southeast Asia, annual proportionate morbidity increased from 50 dengue cases per 1,000 ill returned travelers in nonepidemic years to an average of 159 cases per 1,000 travelers during epidemic years. Dengue can thus be added to the list of diseases for which pretravel advice should include information on relative risk according to season. Also, dengue cases detected at atypical times in sentinel travelers may inform the international community of the onset of epidemic activity in specific areas.

An estimated 100 million cases of dengue fever (DF) and 250,000 cases of dengue hemorrhagic fever (DHF) occur annually (*1*). The past 20 years have seen a dramatic geographic expansion of epidemic DF and DHF from Southeast Asia to the South Pacific Islands, the Caribbean, and the Americans. An increasing number of reports of DF and associated illness among travelers to dengue virus–infected areas parallel the changing epidemiology of dengue in local populations (*2*–*8*). As part of a comprehensive analysis of the spectrum of disease in travelers, the GeoSentinel Surveillance Network, a multifaceted international practice network, has noted that in terms of cumulative case numbers over the past decade, dengue has emerged as a more frequent diagnosis than malaria in ill travelers who have returned from all tropical regions outside of Africa (*9*,*10*).

Dengue accounts for up to 2% of all illness in returned travelers who visit GeoSentinel clinics (*9*). Dengue is also a major cause of hospitalization in febrile returned travelers

*Chaim Sheba Medical Center, Tel Hashomer, Israel; †Tel Aviv University, Tel Aviv, Israel; ‡Statistical Consult, Victoria, British Columbia, Canada; §National University Singapore, Singapore; ¶University of Munich, Munich, Germany; #Toronto General Hospital, Toronto, Ontario, Canada; **University of Toronto, Toronto; ††University of Melbourne, Parkville, Victoria, Australia; ‡‡Royal Melbourne Hospital, Parkville; and §§University of Alabama at Birmingham, Birmingham, Alabama, USA

DOI: 10.3201/eid1407.071412

[1]In addition to the authors, members of the GeoSentinel Surveillance Network who contributed data are: Elizabeth Barnett, Boston University, Boston, Massachusetts, USA; Graham Brown, Royal Melbourne Hospital, Parkville, Victoria, Australia; Giampiero Carosi and Francesco Castelli, University of Brescia, Brescia, Italy; Lin Chen and Mary Wilson, Harvard University, Cambridge, Massachusetts, USA; Bradley Connor, Cornell University, New York, New York, USA; Jean Delmont and Philippe Parola, Hôpital Nord, Marseille, France; Devon Hale and Stefanie Gelman, University of Utah, Salt Lake City, Utah, USA; Elaine Jong and Jean Haulman, University of Washington, Seattle, Washington, USA; Phyllis Kozarsky and Carlos Franco, Emory University, Atlanta, Georgia, USA; Carmelo Licitra, Orlando Regional Health Center, Orlando, Florida, USA; Louis Loutan and Francois Chappuis, University of Geneva, Geneva, Switzerland; Poh Lian Lim, Tan Tock Seng Hospital, Singapore; Michael Lynch, Fresno International Travel Medical Center, Fresno, California, USA; Susan McLellan, Tulane University, New Orleans, Louisiana, USA; Prativa Pandey, CIWEC Clinic Travel Medicine Center, Kathmandu, Nepal; Cecilia Perret, Pontificia Universidad Católica de Chile, Santiago, Chile; Bradley Sack and Robin McKenzie, Johns Hopkins University, Baltimore, Maryland, USA; Hiroko Sagara, Yokohama Municipal Citizen's Hospital, Yokohama, Japan; Marc Shaw, Worldwise Travellers Health and Vaccination Centre, Auckland, New Zealand; Robert Steffan and Patricia Schlagenhauf, University of Zurich, Zurich, Switzerland; William Stauffer and Patricia Walker, University of Minnesota, Minneapolis, Minnesota, USA; and Murray Wittner, Albert Einstein School of Medicine, Bronx, New York, USA.

(*7*,*11*). Prospective seroconversion studies have estimated the attack rate of DF in travelers to the tropics to be 2.9% in Dutch travelers who spent 1 month in Asia (*12*); the seroconversion rate was 6.7% among Israelis who traveled for an average of 5 months (*13*).

We report year-to-year variability, patient characteristics, travel exposures, and region/country specific proportionate illness rates due to dengue in 522 returned travelers. Our sample, collected over a decade, was also of sufficient size to examine the seasonality of dengue in travelers by region. Finally, the use of travelers as sentinels can help provide timely information to the international community about the onset of dengue outbreaks in disease-endemic areas.

Methods

GeoSentinel Surveillance Network

GeoSentinel sites are specialized travel/tropical medicine clinics on 6 continents staffed by clinicians who are recruited on the basis of demonstrated training, experience, and publication in travel and tropical medicine literature. They contribute clinician-based information on all ill travelers seen, including travel history (additional detail is available from www.geosentinel.org) (*9*,*14*). The sites that account for most patient intake are within academic centers; several smaller volume sites (almost all with current academic affiliation) are in freestanding locations. The intake at sites reflects a mixed population of tertiary care and self-referred patients. Some sites are restricted to outpatients, and no one site limits its entire practice to ill travelers. To be eligible for inclusion in the GeoSentinel database, patients must have crossed an international border and be seeking medical advice at a GeoSentinel clinic for a presumed travel-related illness. Anonymous surveillance data that cannot be linked to an individual patient are entered into an SQL database at a central data center. Final diagnoses reported by physicians are used to assign diagnostic codes from a standardized list of >500 etiologic or syndromic diagnoses (*9*).

Inclusion/Exclusion Criteria

All returning travelers who reported to a GeoSentinel site in their current country of residence from October 1, 1997, to March 1, 2006, were eligible for analysis. Many GeoSentinel sites also enter data separately on immigrants with no other travel but the initial immigration trip. None of the patients in this immigrant dataset had a diagnosis of dengue acquired in the country of origin. The current study is restricted to traditional travelers, which also includes immigrants who subsequently traveled from their current country of residence.

Patients were excluded if no confirmed or probable diagnosis was reported. A case of travel-associated dengue was defined per current annual surveillance reports (*15*–*17*), which consider both probable and confirmed cases of dengue (*18*). A case of travel-associated dengue was defined as laboratory-diagnosed dengue in a resident of a non–dengue-endemic area who has traveled to a dengue-endemic area in the 14 days before symptom onset. Laboratory-diagnosed dengue was determined by isolation of dengue virus, virus antigen, or viral RNA, or a serum sample positive for either immunoglobulin (Ig) M or a very high titer of IgG by ELISA. All sites use best available reference diagnostics for their respective countries, which may include well-characterized commercial kits. GeoSentinel criteria for the diagnosis of malaria have been reported (*19*).

Statistical Analysis

Analysis of dengue reports over time was based on proportionate morbidity (the number of patients with dengue fever as a proportion of the number of ill returned travelers visiting a GeoSentinel clinic in that month). Analysis of annual and monthly cycles was based on monthly proportionate morbidity aggregated over all years of data included in the analysis. Patients who were reported as having dengue were compared with all other ill returned travelers in GeoSentinel. A subanalysis, comparing dengue patients with malaria patients, was also performed. We used χ^2 or Fisher exact test as appropriate with a 2-sided significance level of 0.05. Data analysis was performed by using SAS statistical package version 9 (SAS Institute, Cary, NC, USA).

Results

Among ill returned travelers seen at GeoSentinel sites from October 1997 through February 2006, 24,920 met the criteria for analysis. Of these, 522 (2.1%) had a diagnosis of travel-related dengue fever, including 12 patients with dengue hemorrhagic fever or dengue shock syndrome. Of the 522 cases of dengue reported in this study, 68% were seen after travel to Asia, 15% after travel to Latin America, 9% after travel to the Caribbean, 5% after travel to Africa, and 2% after travel to Oceania (Table 1). The countries with the largest number of cases reported among returned travelers were Thailand (154), India (66), Indonesia (38), and Brazil (22).

Annual Trends in Travel-related Dengue and Changes during Regional Epidemics

A comparison of the annual trends in illness from dengue as a proportion of all diagnoses in ill returned travelers showed sustained increases in dengue proportionate morbidity, represented by peaks that are both high and broad in 1998 and 2002. There was also a narrow peak in October 2003 and an increase in late 2005 (Figure 1). When dengue reports were segregated by region, the increases in 1998 and 2002 were found entirely in travelers to Southeast Asia;

Table 1. Dengue and malaria diagnoses as a proportion of all morbidity in ill returned travelers according to region or country of acquisition

Region* or country of exposure	No. ill returned travelers with dengue	No. ill returned travelers with malaria	Total no. ill returned travelers	Dengue proportionate morbidity†	Malaria proportionate morbidity†
Southeast Asia	264	103	3,694	71	28
Thailand	154	9	1,523	101	5
Indonesia	38	53	652	58	81
South Central Asia	90	70	3,303	27	21
India	66	57	2,119	31	27
Caribbean	47	14	1,470	32	9
South America	40	49	2,427	16	20
Brazil	22	12	685	32	18
Central America	37	27	1,867	20	14
Africa	25	1,216	7,231	3	168
Sub-Saharan Africa	23	1,201	6,201	4	194
Oceania	11	91	303	36	300
Other‡ or multiple regions of exposure	7	23	4,443	2	5
Country missing	1	12	182	5	66
Total	522	1,605	24,920	21	64

*Regions defined per (*9*).
†Proportionate morbidity expressed per 1,000 ill returned travelers seen at GeoSentinel clinics.
‡No cases were acquired in Canada, United States, Western Europe, Japan, or Australia.

for 2003, in travelers to South Central Asia; and for 2005, in travelers to South Central Asia and Indonesia. These increases correspond to known epidemic years within local populations for those regions (*20*,*21*).

Since travel-related dengue was found to originate most commonly in Southeast Asia, more detailed analysis could be performed for that region. Dengue proportionate morbidity among ill returned persons who had traveled to Southeast Asia, which was a mean of 71 per 1,000 during the cumulative 1997–2006 period (Figure 2, panel A), was 159 cases per 1,000 ill returned travelers during 1998 and 2002 taken together (Figure 2, panel A); proportionate morbidity reached a peak of >200 cases per 1,000 ill returned travelers during June and July. Of the 264 Southeast Asian cases, 154 that were acquired in Thailand could be plotted separately (Figure 2, panel B). Dengue proportionate morbidity among ill persons who had traveled to Thailand, which was an average of 101 cases per 1,000 during the cumulative 1997–2006 period (Figure 2, panel B), was 257 cases per 1,000 ill returned travelers during 1998 and 2002 taken together (Figure 2, panel B) and was >500 cases per 1,000 ill returned travelers during the peak month of June (i.e., more than half of all ill travelers returning from Thailand had dengue).

Seasonality of Travel-associated Dengue

Figure 3 shows month-by-month dengue cases as a proportion of all illness in ill returned travelers during the study period for each region separately. For Southeast Asia, dengue cases generally peaked in June and September in typical nonepidemic years. However, an examination of the outbreak years of 1998 and 2002 showed that seasonal patterns changed markedly when compared with nonoutbreak years; excess cases were seen for every month except January, and a high and sustained peak occurred from April through August (Figure 2, panel A). In Thailand, during the outbreak years, proportionate morbidity exceeded the mean 1997–2006 proportionate morbidity (Figure 2, panel B) for all months except January. Notably, the major peak of illness began in April, a time of minimal dengue activity in nonoutbreak years. The major epidemic peak in sentinel travelers preceded the epidemic pattern in the local population during 1998 and 2002, as reflected in Thai reports to the World Health Organization (*20*).

An examination of seasonality in travelers for other regions (Figure 3) showed that dengue cases were higher from September through December in South Central Asia

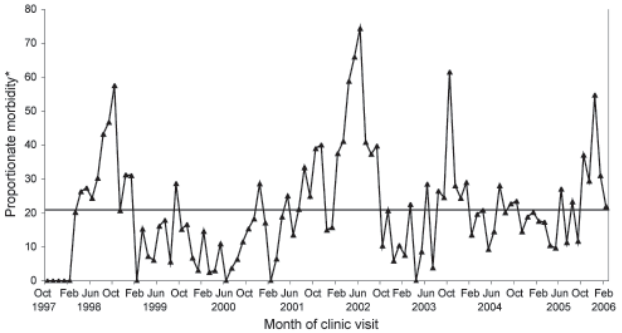


Figure 1. Dengue fever in returned travelers as a proportion of monthly morbidity in all ill returned travelers to all regions of the world. Cumulative proportionate morbidity of 21 per 1,000 ill returned travelers (522 dengue reports among 24,920 ill travelers from October 1997 through February 2006) is shown by the horizontal line. *Proportionate morbidity is expressed as number of dengue cases per 1,000 ill returned travelers.

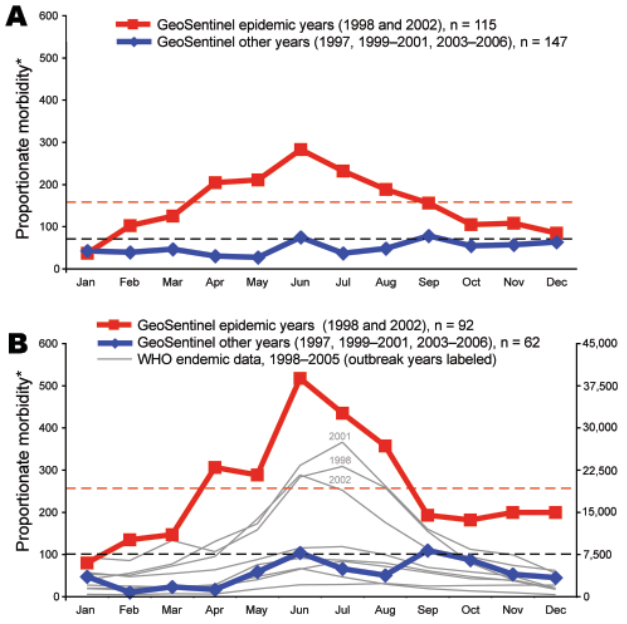


Figure 2. Changes in dengue morbidity during regional epidemics. Heavy red and blue lines show dengue in returned travelers as a proportion of monthly morbidity in all ill returned travelers to Southeast Asia (A) and Thailand (B) during the epidemic years of 1998 and 2002 (red lines) and during all other nonepidemic years (blue lines). Black horizontal dashed lines represent mean proportionate morbidity over all months for that area during the cumulative 1997–2006 period in travelers; red horizontal dashed lines represent mean proportionate morbidity over all months during the 2 outbreak years (1998 and 2002) in travelers. Each gray line in panel B tracks month-by-month reports to the World Health Organization (WHO) of the total number of dengue cases in the endemic Thai population for a single year from 1998–2005. *Proportionate morbidity is expressed as number of dengue cases per 1,000 ill returned travelers.

(especially in India, which accounted for most of our cases in South Central Asia; Table 1). A sharp and consistent major peak can be seen each October throughout the study period. This closely tracks the monthly pattern in the Indian population during 2003–2006, years for which robust Indian national data are available (*21*). The number of dengue cases in travelers was higher from August through December in the Caribbean; it was highest in March in South America, especially in Brazil, which accounted for most of our cases in South America (Table 1). This peak is also consistently seen in the Brazilian population (*22*) each year. No evidence of a strong seasonal pattern was found in travelers to Central America and Africa, although the numbers of travelers to these regions in our sample were small.

Characteristics of Travelers with Dengue

The characteristics of travelers with dengue are compared in Table 2 with the characteristics of those with all other diagnoses. The mean age of dengue patients was 33.8 years; the male:female ratio was 1.17:1. Of the patients studied, 69.3% were traveling only for tourism, and the median trip duration was <28 days. Most of the dengue case-patients (61%) had a pretravel encounter, a significantly higher percentage than for ill returned travelers without dengue (53%; p<0.005). Dengue is overwhelmingly a disease of young adults 18–44 years of age. As expected, due to the short incubation period, >75% of dengue case-patients sought treatment within 2 weeks after return. In addition, significantly more dengue patients were hospitalized (24% vs. 6%; p<0.001), a level similar to the 25% rate reported in a study of European travelers (*4*).

Comparison of Dengue and Malaria Patient Characteristics

During the study period, 1,605 (6.4%) ill returned travelers reported to GeoSentinel had been given a diagnosis of malaria. A comparison of the characteristics of travelers with dengue and those with malaria shows some important differences. Unlike dengue, which affects both sexes almost equally, malaria is more common in male travelers (*11*,*23*). Patients with malaria were less likely to have a recorded pretravel encounter. Duration of travel (median 34 days) was significantly longer than for travelers returning with dengue (median 28 days; p<0.05), although the difference was not large. Malaria was much more common in

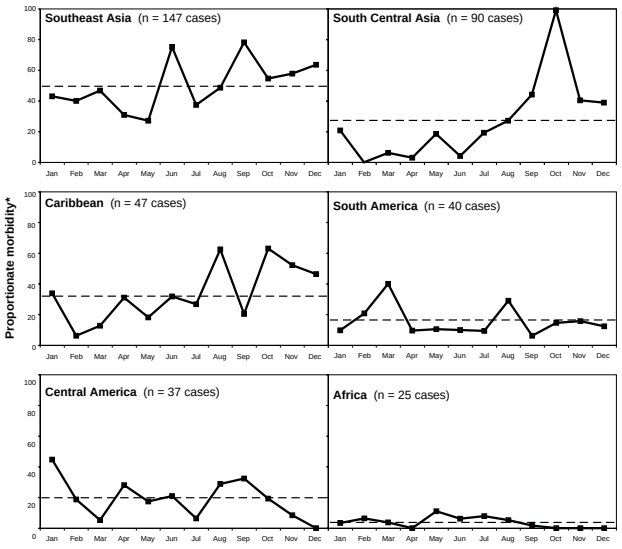


Figure 3. Seasonality of dengue in returned travelers by region. Dengue in returned travelers is shown as a proportion of monthly morbidity in all ill returned travelers to each region. Horizontal dashed lines represent the mean proportionate morbidity over all months for that region during the cumulative 1997–2006 period in travelers. Data for Southeast Asia exclude the outbreak years of 1998 and 2002. *Proportionate morbidity is expressed as number of dengue cases per 1,000 ill returned travelers.

first- or second-generation immigrants visiting friends and relatives (Table 2).

Overall, the proportion of travelers with dengue in the GeoSentinel database (21 cases per 1,000 ill returned travelers) was less than the proportion seen with malaria (64 cases per 1,000 ill returned travelers; Table 1). This finding, however, was mostly due to the disproportionate numbers of travelers returning ill from Africa where malaria is highly prevalent (168 cases per 1,000 ill returned travelers) and where dengue is rare (3 cases per 1,000 ill returned travelers). A similar situation applies to Oceania where malaria (300 cases per 1,000 ill returned travelers) is significantly more frequent than dengue fever (36 cases per 1,000 ill returned travelers). For other regions, the proportionate morbidity due to dengue was higher than that due to malaria, except for South America, where proportionate morbidity was approximately equal (20 cases vs. 16 cases per 1,000 ill returned travelers).

Analysis of travel to several countries was possible. Dengue proportionate morbidity (101 cases per 1,000 ill returned travelers) was dramatically higher than that for malaria (5 cases per 1,000 ill returned travelers) in travelers returning from Thailand and exceeded that for malaria in travelers returning from Brazil and India.

Discussion

Data collected longitudinally over a decade by the GeoSentinel Surveillance Network have allowed us to examine month-by-month illness from a sample of 522 patients with dengue (as a proportion of all diagnoses among 24,920 ill returned travelers) seen at our 33 surveillance sites. Travel-related dengue demonstrates defined seasonality for some regions (Southeast Asia, South Central Asia, the Caribbean, and South America; Figure 3). Although discrete peaks are present, the number of cases from the Caribbean and South America is relatively small. A June peak of travel-related dengue was previously reported in a small sample of 75 Swedish travelers to Thailand (using imported cases from 1998–1999) (*3*). Several vector-borne diseases, such as malaria (*24*) and Japanese encephalitis (*25*), are known to exhibit seasonality in local populations, but no firm data exist on whether this pertains to travelers' risk. Our find-

Table 2. Demographic characteristics and type of travel for ill returned travelers with dengue, malaria, or any other diagnosis

Characteristic	Ill returned travelers with dengue	Ill returned travelers with malaria	Ill returned travelers without dengue or malaria	Total ill returned travelers
No. cases	522	1,605	22,793	24,920
Age group, %*				
<18 y	1.3	5.6	3.9	3.9
18–44 y	79.2	68.4	69.1	69.3
45–55 y	12.4	17.2	14.7	14.7
≥56 y	7.1	8.9	12.4	12.0
Female sex, %*	46.1	30.1	49.7	48.3
Pretravel encounter, %*				
Yes	61.3	42.8	53.6	53.1
No	28.6	43.7	33.6	34.1
Unknown	10.1	13.5	12.8	12.8
Inpatient, %*	24.8	50.3	6.6	9.8
Duration of travel, d*				
25% travelers	14 d	21 d	14 d	14 d
50% travelers	28 d	34 d	28 d	28 d
75% travelers	67 d	95 d	132 d	123 d
Time from travel to symptoms, %*				
<2 week	75.5	53.3	41.8	43.3
≥2 week	24.5	46.7	58.2	56.7
Patient classification %*				
Immigrant	7.9	35.1	13.6	14.9
Temporary visitor	4.2	2.2	1.6	1.7
Expatriate	8.4	13.7	10.0	10.2
All other travelers	79.5	48.4	74.5	73.1
Reason for recent travel, %*				
Tourism	69.3	29.1	60.3	58.5
Business	10.5	16.0	14.0	14.1
Research/education or Missionary/volunteer	10.2	14.5	12.7	12.8
Visiting friends or relatives†	9.8	40.1	12.5	14.2

*Significant differences in travelers with dengue vs. malaria ($p<0.05$).
†1st or 2nd generation immigrant originally from a low-income country now living in a high-income country, visiting friend and relatives in the country of the family's origin.

ings on the seasonality of dengue in travelers benefits those advising prospective travelers, as well as those formulating possible diagnoses in ill returned travelers. Consequently, travelers who have had a previous episode of dengue might want to avoid peak dengue transmission times at a particular destination to minimize the risk for developing dengue hemmorhaghic fever, which preferentially affects those with previous dengue infection (*26*). For example, the February–March peak in Brazil coincides with Carnaval (annual festival marking the beginning of Lent). Nevertheless, in dengue-endemic regions, risk exists year round, and travelers should always be counseled on personal protection measures against arthropods.

Rainy seasons vary by country and, in many cases, vary regionally within countries. Because of these geographic variations in the rainy season, we have avoided the temptation to over generalize about relationships between rainfall and dengue incidence (Figure 3). Although GeoSentinel would not be likely to receive reports from outbreaks of dengue that are restricted to regions of a country not frequented by travelers, most substantial outbreaks do eventually spread widely (*27*). In this analysis, proportionate morbidity always compares the number of dengue cases with all ill travelers seen at GeoSentinel clinics during a particular month. This type comparison ensures that the variation in the absolute number of travelers to a particular destination at different times of year do not distort the results.

The natural, and to a large extent unexplained, year-to-year oscillations of dengue cases in local populations have been described in some countries (*27*–*29*). In travelers, this has not been examined over a long period in such a sizeable dataset, while simultaneously comparing regions of the world (*30*). In each of the epidemic years 1998 and 2002 in Southeast Asia, the usual pattern of seasonality changed with an excess of cases throughout the whole year. The outbreak was heralded initially by an excess of cases beginning in February with a dramatic upsurge in April (Figure 2, panel A), well ahead temporally and in magnitude when compared with the usual initial peak month of June. When the 1998 pattern in travelers recurred in early 2002, it led to the immediate hypothesis that this change of seasonality would once again herald an epidemic year. In April 2002, GeoSentinel alerted the international community when it posted a notice of the increase in travel-related dengue from Thailand online (*31*). Official surveillance data from local populations were not immediately available to the international community. Data reported later by Thai authorities to the World Health Organization confirmed the observation (*20*). A retrospective report published in 2004 also noted an April 2002 surge in dengue cases among German travelers to Thailand (*32*). The increase in dengue cases in returned travelers from South Central Asia in 2003 was also evident before official surveillance data were available. This increase reinforces the usefulness of sentinel surveillance in travelers. For example, travelers' malaria has identified new foci of infection in the Dominican Republic (*33*) and the Bahamas (*34*). Because the number of travelers to areas with epidemics may be small and some epidemics may occur in parts of a country that are not visited by travelers, we are not proposing sentinel surveillance as a definitive and uniquely sensitive tool for detection of all disease outbreaks. A 2001 outbreak in Thailand apparently did not affect travelers (Figure 2, panel B), as it was not associated with a peak in reports to GeoSentinel. Nevertheless, the traveling population can give timely, very specific indicators.

Our data on the high frequency of dengue in travelers to Southeast Asia and the Caribbean and its rarity in travelers to Africa are in agreement with previous smaller samples such as those from a regional European surveillance network (TropNetEurop), which examined 238 returning travelers with dengue over a 3-year period (1999–2001) (*4*). In comparing proportionate morbidity for dengue between regions, rates in travelers to the Caribbean approach those of some parts of Asia and are thus higher than would be expected from overall rates in local populations. These rates likely reflect common travel patterns that may favor more risky locales. A new finding in our report is the high proportionate morbidity in travelers to Oceania, who because of small absolute numbers of travelers to that region, may have been overlooked in earlier studies, which reported only raw numbers of cases.

The limitations of this analysis include those applicable to other published studies that used the GeoSentinel database. The findings can only be generalized to travelers seen in tropical or travel medicine clinics after travel. In general, data do not represent a sample of all returned travelers (e.g., those seen at nonspecialized, primary care practices, where milder and self-limited manifestations of dengue that might not be recognized as such, would occur with greater frequency). The more severely ill patients that do seek treatment at specialized clinics such as GeoSentinel sites will likely have higher hospitalization rates than the overall population with dengue infection. Patients may also seek treatment at GeoSentinel sites and not return for follow-up definitive diagnostic serology when faced with the inconvenience and cost of serologic evaluation of a self-limited illness, particularly when symptoms have resolved. Dengue has a short incubation period; many patients may have the disease while still traveling. Nevertheless, the uncaptured cases are not likely to have a different pattern of geographic acquisition than those that are included.

In conclusion, current data serve as a reference for the seasonality of dengue for several regions of the world. Dengue can be added to the list of diseases for which pretravel advice can include information on relative risk according to season of travel to a particular destination.

Further, the season of travel can aid the clinician in assessing the relative likelihood of dengue in an ill returned traveler with a nonspecific febrile illness. Travelers may be sentinels able to rapidly inform the international community about the onset of epidemics in disease-endemic areas. Effective malaria chemoprophylaxis and strategies for personal protection against night-feeding malaria vectors are already available. Dengue is a risk for all tourists equally without respect to gender, pretravel preparation, or duration of travel. Even with good pretravel advice, all healthcare providers can do is recommend mosquito precautions. The usual preventive measure for an infectious disease is vaccination. Because personal protection against the day-feeding dengue vectors is so problematic, there is an urgent need for a dengue vaccine**.**

Acknowledgments

We are indebted to Elena Axelrod and the staff at each GeoSentinel site for data management and to Adam Plier for ongoing administrative and graphical support.

GeoSentinel, the Global Surveillance Network of the International Society of Travel Medicine, is supported by Cooperative Agreement U50/CI000359 from the Centers for Disease Control and Prevention.

Dr Schwartz is the head of the Center for Geographic Medicine at the Chaim Sheba Medical Center and the Sackler School of Medicine, Tel Aviv University, Israel. His main research interests are travel and tropical medicine.

References

1. Guzman MG, Kouri G. Dengue: an update. Lancet Infect Dis. 2002;2:33–42. DOI: 10.1016/S1473-3099(01)00171-2
2. Wilder-Smith A, Schwartz E. Dengue in travelers. N Engl J Med. 2005;353:924–32. DOI: 10.1056/NEJMra041927
3. Lindbäck H, Lindbäck J, Tegnell A, Janzon R, Vene S, Ekdahl K. Dengue fever in travelers to the tropics, 1998 and 1999. Emerg Infect Dis. 2003;9:438–42.
4. Jelinek T, Mühlberger N, Harms G, Corachán M, Grobusch MP, Knobloch J, et al. European Network on Imported Infectious Disease Surveillance. Epidemiology and clinical features of imported dengue fever in Europe: sentinel surveillance data from TropNetEurop. Clin Infect Dis. 2002;35:1047–52. DOI: 10.1086/342906
5. Schwartz E, Mendelson E, Sidi Y. Dengue fever among travelers. Am J Med. 1996;101:516–20. DOI: 10.1016/S0002-9343(96)00278-1
6. Badiaga S, Barrau K, Brouqui P, Durant J, Malvy D, Janbon F, et al. Infectio-Sud Group. Imported dengue in French university hospitals: a 6-year survey. J Travel Med. 2003;10:286–9.
7. O'Brien DP, Leder K, Matchett E, Brown GV, Torresi J. Illness in returned travelers and immigrants/refugees: the 6-year experience of two Australian infectious diseases units. J Travel Med. 2006;13:145–52. DOI: 10.1111/j.1708-8305.2006.00033.x
8. Bottieau E, Clerinx J, Schrooten W, Van den Enden E, Wouters R, Van Esbroeck M, et al. Etiology and outcome of fever after a stay in the tropics. Arch Intern Med. 2006;166:1642–8. DOI: 10.1001/archinte.166.15.1642
9. Freedman DO, Weld LH, Kozarsky PE, Fisk T, Robins R, von Sonnenburg F, et al. GeoSentinel Surveillance Network. Spectrum of disease and relation to place of exposure among ill returned travelers. N Engl J Med. 2006;354:119–30. DOI: 10.1056/NEJMoa051331
10. Wilson ME, Weld LH, Boggild A, Keystone JS, Kain KC, von Sonnenburg F, et al. GeoSentinel Surveillance Network. Fever in returned travelers: results from the GeoSentinel Surveillance Network. Clin Infect Dis. 2007;44:1560–8. DOI: 10.1086/518173
11. Stienlauf S, Segal G, Sidi Y, Schwartz E. Epidemiology of travel-related hospitalization. J Travel Med. 2005;12:136–41.
12. Cobelens FG, Groen J, Osterhaus AD, Leentvaar-Kuipers A, Wertheim-van Dillen PM, Kager PA. Incidence and risk factors of probable dengue virus infection among Dutch travellers to Asia. Trop Med Int Health. 2002;7:331–8. DOI: 10.1046/j.1365-3156.2002.00864.x
13. Potasman I, Srugo I, Schwartz E. Dengue seroconversion among Israeli travelers to tropical countries. Emerg Infect Dis. 1999;5:824–7.
14. Freedman DO, Kozarsky PE, Weld LH, Cetron MS. GeoSentinel: the global emerging infections sentinel network of the International Society of Travel Medicine. J Travel Med. 1999;6:94–8. DOI: 10.1111/j.1708-8305.1999.tb00839.x
15. Centers for Disease Control and Prevention. Travel-associated dengue—United States, 2005. MMWR Morb Mortal Wkly Rep. 2006;55:700–2.
16. Centers for Disease Control and Prevention. Travel-associated dengue infections—United States, 2001–2004. MMWR Morb Mortal Wkly Rep. 2005;54:556–8.
17. Centers for Disease Control and Prevention. Imported dengue—United States, 1999 and 2000. MMWR Morb Mortal Wkly Rep. 2002;51:281–3.
18. Rigau-Perez JG, Laufer MK. Dengue-related deaths in Puerto Rico, 1992–1996: diagnosis and clinical alarm signals. Clin Infect Dis. 2006;42:1241–6. DOI: 10.1086/501355
19. Leder K, Black J, O'Brien D, Greenwood Z, Kain KC, Schwartz E, et al. Malaria in travelers: a review of the GeoSentinel surveillance network. Clin Infect Dis. 2004;39:1104–12. DOI: 10.1086/424510
20. World Health Organization Regional Office for South East Asia. Dengue/DHF: seasonal trends of DF/DHF in selected countries in SEA Region. [cited 2007 Oct 26]. Available from http://www.searo.who.int/EN/Section10/Section332_9482.htm
21. Chakravarti A, Kumaria R. Eco-epidemiological analysis of dengue infection during an outbreak of dengue fever, India. Virol J. 2005;2:32 [cited 2008 May 29]. Available from http://www.virologyj.com/content/2/1/32
22. Siqueira JB Jr, Martelli CM, Coelho GE, Simplicio AC, Hatch DL. Dengue and dengue hemorrhagic fever, Brazil, 1981–2002. Emerg Infect Dis. 2005;11:48–53.
23. Genton B, D'Acremont V. Clinical features of malaria in returning travelers and migrants. In: Schlagenhauf P, editor. Travelers' malaria. Hamilton: BC, Ontario, Canada, Decker; 2001. p. 371–92.
24. World Health Organization. Country list: yellow fever vaccination requirements and recommendations; and malaria situation. In: International travel and health: situation as of 1 January 2007. Geneva: World Health Organization; 2007.
25. Halstead SB, Jacobson J. Japanese encephalitis. Adv Virus Res. 2003;61:103–38. DOI: 10.1016/S0065-3527(03)61003-1
26. Halstead SB. Dengue. Curr Opin Infect Dis. 2002;15:471–6.
27. Cummings DA, Irizarry RA, Huang NE, Endy TP, Nisalak A, Ungchusak K, et al. Travelling waves in the occurrence of dengue haemorrhagic fever in Thailand. Nature. 2004;427:344–7. DOI: 10.1038/nature02225
28. Wearing HJ, Rohani P. Ecological and immunological determinants of dengue epidemics. Proc Natl Acad Sci U S A. 2006;103:11802–7. DOI: 10.1073/pnas.0602960103

29. Bartley LM, Donnelly CA, Garnett GP. The seasonal pattern of dengue in endemic areas: mathematical models of mechanisms. Trans R Soc Trop Med Hyg. 2002;96:387–97. DOI: 10.1016/S0035-9203-(02)90371-8
30. Schwartz E, Moskovitz A, Potasman I, Peri G, Grossman Z, Alkan ML. Changing epidemiology of dengue fever in travelers to Thailand. Eur J Clin Microbiol Infect Dis. 2000;19:784–6. DOI: 10.1007/s100960000388
31. Freedman DO, Kozarsky P, Schwartz E. Thailand: out of season dengue outbreak in travellers to Koh Phangan. ProMed. April 26, 2002 [cited 2008 May 19]. Available from http://www.promedmail.org, archive number: 20020426.4039.
32. Frank C, Schöneberg I, Krause G, Claus H, Ammon A, Stark K. Increase in imported dengue, Germany, 2001–2002. Emerg Infect Dis. 2004;10:903–6.
33. Centers for Disease Control and Prevention. Transmission of malaria in resort areas—Dominican Republic, 2004. MMWR Morb Mortal Wkly Rep. 2005;53:1195–8.
34. Centers for Disease Control and Prevention. Malaria—Great Exuma, Bahamas, May–June 2006. MMWR Morb Mortal Wkly Rep. 2006;55:1013–6.

Address for correspondence: David O. Freedman, William C. Gorgas Center for Geographic Medicine, Division of Infectious Diseases, Department of Medicine, University of Alabama at Birmingham, 1530 3rd Ave S, BBRB 203, Birmingham, AL 35294-2170, USA; email: freedman@uab.edu

A Prospective Study of Etiology of Childhood Acute Bacterial Meningitis, Turkey

Mehmet Ceyhan,* Inci Yildirim,* Paul Balmer,† Ray Borrow,† Bunyamin Dikici,‡ Mehmet Turgut,§ Nese Kurt,§ Aysel Aydogan,¶ Cigdem Ecevit,¶ Yasar Anlar,# Ozlem Gulumser,# Gonul Tanir, Nuran Salman,†† Nezahat Gurler,†† Nevin Hatipoglu,†† Mustafa Hacimustafaoglu,‡‡ Solmaz Celebi,‡‡ Yavuz Coskun,§§ Emre Alhan,¶¶ Umit Celik,¶¶ Yildiz Camcioglu,†† Gulten Secmeer,* Deniz Gur,## and Steve Gray†**

Determination of the etiology of bacterial meningitis and estimating cost of disease are important in guiding vaccination policies. To determine the incidence and etiology of meningitis in Turkey, cerebrospinal fluid (CSF) samples were obtained prospectively from children (1 month–17 years of age) with a clinical diagnosis of acute bacterial meningitis. Multiplex PCR was used to detect DNA evidence of *Streptococcus pneumoniae*, *Haemophilus influenzae* type b (Hib), and *Neisseria meningitidis*. In total, 408 CSF samples were collected, and bacterial etiology was determined in 243 cases; *N. meningitidis* was detected in 56.5%, *S. pneumoniae* in 22.5%, and Hib in 20.5% of the PCR-positive samples. Among *N. meningitidis*–positive CSF samples, 42.7%, 31.1%, 2.2%, and 0.7% belonged to serogroups W-135, B, Y, and A, respectively. This study highlights the emergence of serogroup W-135 disease in Turkey and concludes that vaccines to prevent meningococcal disease in this region must provide reliable protection against this serogroup.

Acute bacterial meningitis is one of the most severe infectious diseases, causing neurologic sequelae and accounting for an estimated 171,000 deaths worldwide per year (*1*,*2*). Although most disease occurs in infants, the societal impact is also important because of the continued high incidence in healthy older children and adolescents. Despite many new antibacterial agents, bacterial meningitis fatality rates remain high, with reported rates between 2% and 30% (*3*,*4*). Furthermore, permanent sequelae, such as epilepsy, mental retardation, or sensorineural deafness are observed in 10%–20% of those who survive (*5*,*6*).

The 3 most common etiologic agents are *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, and *Neisseria meningitidis*, which account for 90% of reported cases of acute bacterial meningitis in infants and children >4 weeks of age (*7*,*8*). Hib meningitis is a disease affecting primarily young children; most of the cases occur in children 1 month to 3 years of age (*3*,*8*). The use of Hib conjugate vaccines has reduced the incidence of, or even virtually eliminated, invasive Hib disease in some industrialized countries (*7*,*8*). *S. pneumoniae* is a major cause of childhood bacterial meningitis in countries where Hib disease has been eliminated by vaccination (*9*). It is the second most frequently reported cause of septic meningitis in some European and sub-Saharan African countries, after meningococcal cases (*4*,*9*).

N. meningitidis is now considered to be the leading cause of bacterial meningitis in many regions of the world, causing an estimated 1.2 million cases of bacterial meningitis and sepsis worldwide each year (*10*,*11*). Meningococci are classified into 13 serogroups based on the antigenic properties of their capsular polysaccharide; however, nearly all disease is caused by 5 serogroups: A, B, C, W-135, and Y. The epidemiology of *N. meningitidis* varies by sero-

*Hacettepe University, Ankara, Turkey; †Health Protection Agency North West, Manchester, United Kingdom; ‡Dicle University, Diyarbakir, Turkey; §Firat University, Elazig, Turkey; ¶Dr. Behcet Uz Children's Hospital, Izmir, Turkey; #Mayis University, Samsun, Turkey; **Sami Ulus Children's Hospital, Ankara; ††Istanbul University, Istanbul, Turkey; ‡‡Uludag University, Bursa, Turkey; §§Gaziantep University, Gaziantep, Turkey; ¶¶Cukurova University, Adana, Turkey; and ##Pediatric Microbiology Laboratory, Ankara

DOI: 10.3201/eid1407.070938

group; currently, serogroups A, B, and C account for >90% of meningococcal disease worldwide (*12*). However, the epidemiologic landscape is constantly changing, and with increasing international travel and cross-border migration, the epidemiology of this disease will remain dynamic. Currently, serogroups A and C predominate throughout Asia and Africa, whereas serogroups B and C are responsible for most cases in Europe and North America (*11*,*13*–*18*). In several countries, including the United States, the proportion of disease caused by serogroup Y has increased over the past decade, where it now accounts for approximately one third of meningococcal cases (*19*). Serogroup W-135 has also recently emerged in some parts of the world, primarily in the Middle East and Africa, in some instances causing large epidemics (*20*).

The annual Hajj pilgrimage to Mecca is a major international event; ≈2 million people from around the world gather in one place, where the extreme crowding provides an ideal environment for transmission of meningococcal carriage. On several occasions, meningococcal disease outbreaks have subsequently spread worldwide by returning pilgrims. A major serogroup A meningococcal disease epidemic occurred in the 1980s, affecting Muslim pilgrims initially, followed by populations in other Middle Eastern and African countries (*21*). After this epidemic, Hajj pilgrims were vaccinated with a bivalent (A and C) meningococcal polysaccharide vaccine before entering Saudi Arabia. With the emergence of serogroup W-135 meningococcal disease among Hajj pilgrims in the Middle East during 2000 and 2001 (*20*), vaccine recommendations for pilgrims were changed to quadrivalent (A, C, W-135, and Y) meningococcal polysaccharide vaccine in 2002 (*22*).

Global surveillance of confirmed meningococcal cases, including surveillance of the diversity of causative strains, is essential to managing disease and developing vaccines. This study was undertaken to determine the current etiology of bacterial meningitis in Turkey, with particular emphasis on serogroup distribution of meningococci. Turkey is a predominantly Muslim country, and as such epidemics originating at the Hajj may have an effect on the national epidemiology. Although limited epidemiologic studies are available, cases of invasive meningococcal disease as well as carriage of serogroup W-135 have been reported in Turkey (*23*–*25*). This finding is in contrast to Western Europe, where the incidence of W-135 disease remains low. Turkey has no surveillance system for bacterial meningitis, and exact rates of meningococcal disease and serogroup distribution are unknown. Reliable surveillance data from countries such as Turkey are vital to understand, and better anticipate, the constantly changing landscape of bacterial meningitis and meningococcal disease.

Materials and Methods

Study Design

From February 16, 2005, through February 15, 2006, active surveillance of acute bacterial meningitis among children admitted to 12 participating hospitals was undertaken. Turkey is divided into 7 geographic areas (Figure 1). Twelve health centers in 9 cities located in all of these 7 geographic regions were selected to represent the population characteristics of the country. Two centers from each of the 3 biggest cities and 1 center from each of the other cities were included. Each health center served as a referral center for its region in the field of pediatric diseases. The centers serve ≈32% of the entire pediatric population of Turkey. Approval was obtained from the ethical committees of the participating centers and Ministry of Health.

In each hospital, suspected cases of acute bacterial meningitis were identified by a pediatrician, based on the following criteria: any sign of meningitis (fever [axillary measurement ≥38°C], vomiting [≥3 episodes in 24 h], headache, meningeal irritation signs [bulging fontanel, Kernig or Brudzinski signs, or neck stiffness]) in children >1 year of age; fever without any documented source; impaired consciousness (Blantyre Coma Scale <4 if <9 months of age and <5 if ≥9 months of age) (*26*); prostration (inability to sit unassisted if ≥9 months of age or breastfeed if <9 months of age) in those <1 year of age; and seizures (other than those regarded as simple febrile seizures with full recovery within 1 h). For each suspected case, demographic data, predominant clinical signs and symptoms, prior history of use of antimicrobial agent, and laboratory results were recorded by using a standardized case report form.

Cerebrospinal fluid (CSF) samples were obtained from all patients <17 years of age with clinical suspected meningitis. Patient samples were included in further analyses

Figure 1. Meningitis cases by geographic region of Turkey. The number of suspected meningitis cases included in the study per region is shown in **boldface**, with the region-specific estimated incidence rate of laboratory-confirmed meningitis (per 100,000 population) shown in parentheses. In total, 408 children were diagnosed with suspected acute bacterial meningitis. Bacterial meningitis was confirmed by PCR, culture, or latex agglutination test in 243 cases. Region-specific incidence rates ranged from 1.0 to 10.9/100,000 population.

if the CSF had 1) >10 leukocytes/mm^3 in the CSF, and/or 2) higher CSF protein levels than normal for the patient's age, and/or 3) lower CSF glucose levels than normal for the patient's age. In addition to these patients, all who had a positive CSF culture, PCR, Gram stain, or antigen detection test result were also included in the study. No neonates (<1 month of age) were included in the study since the pathogens of neonatal meningitis were expected to be different than those of the older case-patients (*3*).

CSF Cultures and Bacterial Isolates

CSF cultures, Gram stain, and latex agglutination tests (Wellcogen Bacterial Antigen Kit, Lenexa, KS, USA) were performed in the local hospitals. A CSF sample (minimum 0.5 mL) from each patient was kept at –20°C until transportation to the Central Laboratory (Hacettepe University, Ankara, Turkey) for PCR analysis. CSF samples and, if available, bacterial isolates were sent to the Central Laboratory, where all isolates were recultured on chocolate and blood agars and grown at 37°C in 5% CO_2. Suspected meningococcal colonies were characterized by Gram stain, oxidase test, and rapid carbohydrate utilization test (Gallerie Pasteur, Pasteur Merieux, Lyon, France). The microbiology laboratory records were cross-checked in each hospital for missing data. The phenotypic determination, based on the antigenic formula (serogroup: serotype:serosubtype) of meningococcal isolates, was performed by standard methods in the Meningococcal Reference Unit, Health Protection Agency, Manchester, United Kingdom (*18*,*20*–*25*,*27*).

DNA Isolation

All samples collected in the Central Laboratory were kept at –80°C and were thawed immediately before each test. Bacterial colonies were suspended in 500 μL sterile double-distilled water and vortexed. Bacterial suspensions and CSF were boiled for 3 min at 100°C, then centrifuged for 5 min at 10,000 × *g*, and the supernatant was retained. DNA concentration was estimated spectrophotometrically, and 15 μL (≈50 ng) was used in each final reaction mixture.

PCR Amplification

For the simultaneous identification of bacterial agents, single tube, multiplex PCR assay was performed. The specific gene targets were *ctrA*, *bex,* and *ply* for *N. meningitidis,* Hib, and *S. pneumoniae,* respectively (*28*). In each assay, the final reaction mixture of 50 μL contained 15 μL (≈50 ng) DNA, 1× PCR buffer, 3 mmol/L $MgCl_2$, 200 μmol/L of each dNTP (AB Gene, Epsom, UK), 0.6 μmol/L of each corresponding oligonucleotide primer (Sigma Aldrich, Seezle, Germany) as described (*28*) and 1 U of Taq polymerase (AB Gene). The PCR was performed by using a DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, CA, USA model 9600) under the following conditions: a first cycle of denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 25 s, 57°C for 40 s, and 72°C for 60 s.

Among the samples positive for *N. meningitidis,* serogroup prediction (A, B, C, W-135, and Y) was based on the oligonucleotides in the *siaD* gene for serogroups B, C, W-135, and Y and in orf-2 of a gene cassette required for serogroup A (*28*). For serogroup determination, amplification reactions (50 μL) contained 15 μL of DNA, 60 mmol/L Tris-HCl (pH 8.8), 17 mmol/L $(NH4)_2SO_4$, 5 mmol/L $MgCl_2$, 0.5 mmol/L of each dNTP, 0.3 μmol/L corresponding oligonucleotides, and 1 U of Taq polymerase. The PCR conditions were as follows: denaturation at 94°C for 3 min, followed by 35 cycles of 92°C for 40 s, 55°C for 30 s, and 72°C for 20 s in a DNA thermal cycler. A final extension cycle at 72°C for 10 min was then performed (*29*).

All amplicons were analyzed by electrophoresis on standard 3% agarose gels and visualized by using UV fluorescence. A negative control consisting of distilled water and a positive control consisting of a reference strain (*S. pneumoniae* ATCC 49613, Hib ATCC 10211, *N. meningitidis* serogroup C L94 5016 also known as C11 [C:16:P1.7–1,1], serogroup A M99 243594 [A:4,21:P1.20,9], serogroup Y M05 240122 [Y:NT:P1.5], serogroup W135 M05 240125 [W135:2a:NST], serogroup B M05 240120 [B:NT:NST]) were analyzed simultaneously.

Statistical Analysis

Continuous variables were compared by the Student *t* test and categorical variables with χ^2 or Fisher exact tests. A 2-tailed p value <0.05 was considered significant. All statistical analysis was performed with SPSS version 11.5 (SPSS Inc, Chicago, IL, USA).

Results

Meningitis Cases

In total, 408 children were hospitalized with a clinical diagnosis of meningitis during the study period (Figure 1), and a CSF sample from each patient was obtained. The distribution of these suspected cases according to the geographic regions was as follows: 109 (26.7%) in Southern Anatolia, 74 (18.1%) in Aegean region, 79 (19.6%) in Central Anatolia, 53 (13.0%) in Marmara region, 43 (8.7%) in Eastern Anatolia, 24 (5.9%) in Black Sea region, and 26 (6.4%) in Mediterranean region. The mean age of the 408 children was 4.8 years (standard deviation 4.1 years), and the boy-to-girl ratio was 1.5:1. Of 408 patients diagnosed with acute bacterial meningitis, 20 (4.9%) died and 14 (5.7%) of these deaths were among patients with laboratory-confirmed cases.

Laboratory-Confirmed Meningitis Cases and Etiology

Of the 408 cases, bacterial meningitis was confirmed by PCR, culture, or latex agglutination test in 243 (59.6%) patients. Regional incidence rates of laboratory-confirmed meningitis were estimated as ranging from 1/100,000 population in the Black Sea region to 10.9/100,000 population in the Southeast Anatolia region (Figure 1). Nationwide, the highest incidence was in children 1–12 months of age and was slightly more common in boys. The boy-to-girl ratio of the confirmed cases was 1.3:1, and the age distribution was as shown in Figure 2.

Overall, the diagnosis of acute bacterial meningitis was confirmed with CSF culture in 41 (17%) of 243 cases, with latex agglutination test in 56 (23%), and with PCR in 243 (100%) (Table). Latex agglutination test was positive in 37 cases for *N. meningitidis*, in 10 cases for Hib, and in 9 cases for *S. pneumoniae*.

Where data were available, 7 (17%) of 41 cases with positive CSF culture and 111 (54.9%) of 202 cases with negative CSF culture had a history of use of antimicrobial agent(s) before lumbar puncture, which may account for the relatively low diagnosis rate by using this technique. *N. meningitidis* was reported in 23 cases, *S. pneumoniae* was reported in 12, and Hib was reported in 6 cases as positive in CSF culture.

Blood culture was positive in 12 (4.9%) of 243 cases—4 each of *N. meningitidis*, Hib, and *S. pneumoniae*. Phenotyping of 21 available isolates indicated W135:2a:P1.5,2 (5 cases), A:21:NT:P1.10 (1 case), B:NT:P1.12,4 (2 cases), B:22:NT:NT (2 cases), B:NT:NT:P1.14 (2 cases), B:15:P1.7,16 (3 cases), B:14:NT:P1.13 (2 cases), B:15:NT:P1.16 (2 cases), X:NT:P1.7,1 (1 case), Y:NT:P1.5:NT (1 case).

PCR analysis was by far the most reliable method of confirming bacterial meningitis, accounting for all confirmed cases with 243 positive results. In these PCR-positive samples, 138 (56.5%) were attributable to *N. meningitidis*, 55 (22.5%) to *S. pneumoniae*, and 50 (20.5%) to Hib (Figure 3). Of the 408 patients, 118 (48.5%) of 243 cases with positive PCR and 96 (58.2%) of 165 cases with negative PCR had received antibacterial drugs in the week before CSF sampling.

In the evaluation of the bacterial agents among the 7 different geographic regions of the country, *N. meningitidis* was the most common cause of acute bacterial meningitis in all regions except the Mediterranean region, located on the southern coast of Turkey. Here *S. pneumoniae* were the prominent bacteria, and *N. meningitidis* were detected in only 2 cases.

Comparison of the incidence of *N. meningitidis*, *S. pneumoniae*, and Hib among different age groups demonstrated that *N. meningitidis* was the prominent bacterial agent causing acute bacterial meningitis, especially in children <7 years of age (Figure 2). The highest incidence was detected during the first year of life for all 3 bacteria.

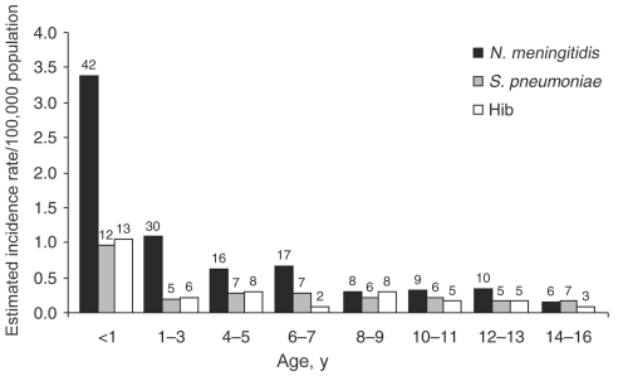


Figure 2. Distribution of bacteria causing childhood acute bacterial meningitis in different age groups. *Neisseria meningitidis* was the most common cause of meningitis, and the highest estimated incidence was in children <1 year of age for all 3 bacteria. The number of cases is indicated above each bar. *S. pneumoniae*, *Streptococcus pneumoniae*; Hib, *Haemophilus influenzae* type b.

CSF findings were recorded for 368 (90.2%) of 408 CSF samples sent to the Central Laboratory. As a mean, in PCR-negative samples, CSF protein level was significantly lower (70.2 vs. 130.4 mg/dL; p = 0.003) and glucose level was significantly higher (55.6 vs. 41.3 mg/dL; p = 0.01) than in PCR-positive samples. Total cell counts were not significantly different in PCR-negative and -positive samples (157.9 and 211.1/mm^3; p>0.05), but polymorphonuclear cell count was significantly higher in PCR-positive samples (8,284.3 vs. 38.5/mm^3; p = 0.001). Among the PCR-positive samples, total cell and polymorphonuclear cell counts were not significantly different between samples positive for *N. meningitidis*, *S. pneumoniae*, and Hib (p>0.05).

Meningococcal Epidemiology

Among the samples that were positive for *N. meningitidis* following PCR analysis, serogroup W-135 was the cause of most infections; 59 (42.7%) cases were serogroup W-135, 43 (31.1%) were serogroup B, 3 (2.2%) were serogroup Y, and 1 (0.7%) was serogroup A. There were no cases with a positive result for serogroup C (this was also

Table. Laboratory confirmation of bacterial meningitis*

Category	PCR positive (n = 243)	PCR negative (n = 165)	Total (n = 408)
CSF culture			
Positive	41	0	41
Negative	202	165	367
Latex agglutination test			
Positive	53	3	56
Negative	190	162	352

*Specimens positive for individual assays and combinations of assays. CSF, cerebrospinal fluid.

the case following analysis of CSF culture-positive samples) and in 32 (23.2%) *N. meningitidis*–positive samples the serogroup could not be determined by the PCR assay.

Analysis by age reveals the greatest meningococcal disease incidence is in children <3 years of age, particularly infants <1 year of age. The numbers of cases caused by the 2 most common *N. meningitidis* serogroups (serogroups W-135 and B) were similar in the most vulnerable age groups (<3 years of age), but W-135 was more common in children 4–16 years of age (Figure 4).

Etiologic and meningococcal serogroup distribution among the different geographic regions is illustrated in Figure 5. *N. meningitidis* serogroup W-135 was more prominent than the other meningococcal serogroups in the Southeast Anatolia, Aegean (Western Turkey), Eastern Anatolia, and Black Sea regions. *N. meningitidis* serogroup B was much more common in the Marmara region (northwestern Turkey), and in the Central region; the numbers of serogroup B and serogroup W-135 cases were similar. The Mediterranean region had 2 *N. meningitidis*–positive samples; both were nongroupable by PCR analysis.

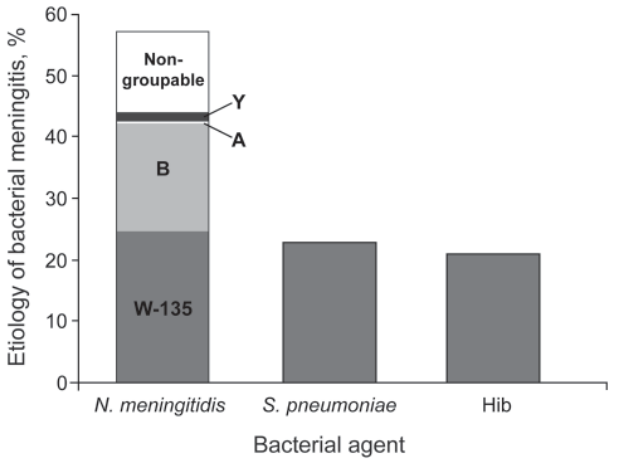


Figure 3. Distribution of etiology of acute bacterial meningitis in Turkey detected by using PCR analysis. Of 243 PCR-confirmed cases, 138 (56.5%) were attributable to *Neisseria meningitidis*, 55 (22.5%) to *Streptococcus pneumoniae*, and 50 (20.5%) to *Haemophilus influenzae* type b (Hib).

Discussion

In this study, 243 cases of laboratory-confirmed acute bacterial meningitis were recorded. Because our study centers provide service to 32% of the population of Turkey, extrapolation from the number of cases recorded suggests that 759 acute bacterial meningitis cases (excluding neonatal cases) per year occur in the whole country. The population of children 1 month through 16 years of age was calculated as 21.6 million. Therefore, the annual incidence of acute bacterial meningitis was estimated as 3.5 cases/100,000/year. Although similar to incidence rates reported from other countries without routine vaccination against *N. meningitidis, S. pneumoniae*, and Hib (*3*,*4*,*10*,*30*), this value likely represents a lower limit estimate of the true disease incidence, given the inherent limitations of hospital-based surveillance. Furthermore, the specific role of these 3 most common bacterial causes of acute bacterial meningitis varies between regions.

An accurate laboratory confirmation of the etiology in acute bacterial meningitis is essential to provide optimal patient therapy, appropriate case contact management, and reasoned public health actions. Prospectively, it also provides information upon which to base decisions regarding immunization programs, especially for countries without routine vaccination against the main acute bacterial meningitis pathogens (*28*,*31*). Although bacterial culture is considered to be the standard method, the negative effect of prior antimicrobial drug use on its sensitivity necessitates nonculture techniques for diagnosis. Among nonculture diagnostic tests, PCR is the most accurate and reliable method, especially among patients with a history of antimicrobial drug use before spinal tap (*32*). This finding was evident in the present study, in which PCR analysis was the most sensitive method, confirming 243 cases (59.6%) among 408 children meeting the case definition for bacterial meningitis (100% of all cases that were confirmed by any method). Using other methods that are more sensitive may increase the rate of laboratory confirmation.

Several reports review the rates of bacterial causes of acute bacterial meningitis from many different countries, based on CSF cultures. Some factors, such as previous antimicrobial drug treatment, interfere with the recovery of microorganisms from CSF. In our study, bacterial isolation was only possible in 41(16.8%) of 243 of confirmed cases. However, the most important factor for this low positive

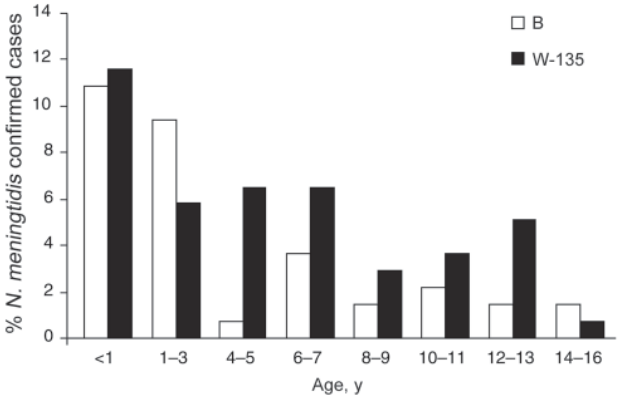


Figure 4. Distribution of predominant *Neisseria meningitidis* serogroups in different age groups. Serogroups W-135 and B caused 42.7% and 31.1% of all meningococcal infections, respectively. W-135 was the most common cause of meningococcal infection in all but 2 age groups analyzed.

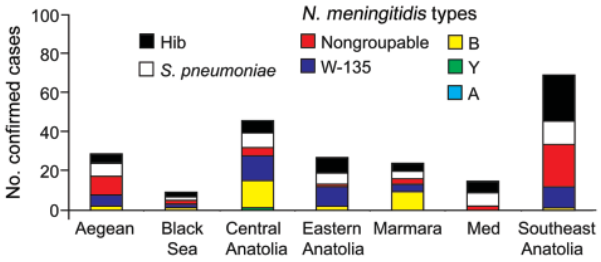


Figure 5. Etiology of confirmed cases of bacterial meningitis in different geographic regions. W-135 was the most prominent *Neisseria meningitidis* serogroup in the Southeast Anatolia, Aegean, Eastern Anatolia, and Black Sea regions. The percentages of cases caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) are also shown.

ratio in culture was likely prior antimicrobial drug use, as 118 case-patients received such treatment before the lumbar puncture (7/41; 17% in culture-positive case-patients and 111 (54.9%) of 202 in culture-negative case-patients). In patients with acute bacterial meningitis, blood cultures can be used in the etiologic diagnosis in up to 80% of cases since the bacteria generally invade meningeal membranes following bacteremia (*3*,*10*,*33*). In our study, however, only 12 (4.9%) of 243 case-patients who had blood culture tests returned positive results. This finding may also be related to the previous antimicrobial drug use.

Previous reports suggested that *S. pneumoniae* and *N. meningitidis* serogroups A and B would be the most common bacteria causing acute bacterial meningitis in Turkey (*25*,*34*–*36*). Serogroup W-135 meningococcus was isolated for the first time in Turkey in an asymptomatic healthy preschool child in 2001 (*23*), and the first patient with meningitis caused by serogroup W-135 was reported in 2003 (*24*). In this study *N. meningitidis,* especially serogroup W-135, was responsible for most of the cases observed, with serogroup B the second most common. Only a small number of serogroup A or Y cases were noted, and no serogroup C cases were observed. These data are in contrast to those from many parts of Europe, where serogroups B and C dominate the epidemiologic landscape. In Turkey, meningococcal disease caused by serogroup W-135 has increased from 1 case in 2003 to 59 cases or 42.7% of all laboratory-confirmed *N. meningitidis* cases in children, in this study, during 2005/06. A dramatic increase in serogroup Y disease has been documented in the United States during the last decade, but this has been over a longer period.

Most of the Turkish population is Muslim, and ≈150,000 pilgrims travel annually to Saudi Arabia for the Hajj. Since 2002, all Turkish pilgrims have received a quadrivalent meningococcal polysaccharide vaccine before travel. Although this vaccine generates a robust immune response against serogroup W-135, in contrast to what has been demonstrated for serogroup C meningococcal conjugate vaccines (*37*), meningococcal polysaccharide vaccines are not thought to reliably prevent asymptomatic carriage. A study from the United States reported that 0.8% of returning vaccinated pilgrims in 2001 were carrying W-135, whereas no pilgrims carried this serogroup upon departure from the United States (*38*). Therefore, the rapid rise in the proportion of cases caused by serogroup W-135 may be attributable to transmission from pilgrims returning from the Hajj carrying this particular serogroup. Although not definitive, this conclusion is further supported by the finding that all serogroup W-135 isolates available for phenotypic characterization were identical to the Hajj-associated clone, W135:2a:P1.5,2 (*20*). Because strains with this serologic profile have not been uniquely associated with the Hajj outbreak (*39*), additional typing data (e.g., multilocus sequence typing or multilocus enzyme electrophoresis), and epidemiologic investigations will be required to support this hypothesis. Therefore, it remains speculative that the increased W-135 disease in Turkey may be caused by spread of Hajj epidemic strain through carriage and transmission by pilgrims.

This study demonstrates the need for good quality, continued surveillance of bacterial meningitis cases, as well as the etiology and epidemiology of the causative bacteria. Only by accurately monitoring meningococcal epidemiology will effective vaccination policies be developed. The bacterial meningitis epidemiologic landscape is not static, and the causative agents change with time and across regions of the world. This study has demonstrated that the relative contribution of serogroup W-135 to the meningococcal disease incidence in Turkey is increasing, which is in contrast to the rest of Europe. Turkey may remain isolated in terms of W-135 disease incidence or it may represent the beginning of epidemiologic change in Eastern Europe. This possibility should be investigated in greater depth and monitored prospectively. Introduction of vaccines can dramatically reduce the meningitis disease incidence, but these vaccines must be targeted against the correct bacteria and, where relevant, the correct bacterial serogroup. The choice of meningococcal conjugate vaccines in Turkey will need to include coverage for serogroup W-135; introduction of such a vaccine would be helpful in protecting the Turkish population from this invasive bacterial meningitis. Moreover, it may also be prudent to switch the meningococcal vaccine used for pilgrims to conjugated vaccine to prevent the carriage of the microorganism by pilgrims.

This study was supported by Novartis Vaccines.

Dr Ceyhan is professor of pediatrics and pediatric infectious diseases at Hacettepe University, Ankara. His areas of interest

include vaccines, epidemiology of vaccine-preventable diseases, and mechanisms of antibacterial resistance.

References

1. World Health Organization. World health report. 2000 [cited 2008 May 1]. Available from http://www.who.int/whr/2000/en/index.html
2. Jodar L, Feavers IM, Salisbury D, Granoff DM. Development of vaccines against meningococcal disease. Lancet. 2002;359:1499–508. DOI: 10.1016/S0140-6736(02)08416-7
3. Feigin RD, Pearlman E. Bacterial meningitis beyond the neonatal period. In: Feigin RD, Demler GJ, Cherry JD, Kaplan SL, editors. Textbook of pediatric infectious diseases. 5th ed. Philadelphia: Saunders; 2004. p. 443–74.
4. Saez-Llorens X, McCracken GH Jr. Bacterial meningitis in children. Lancet. 2003;361:2139–48. DOI: 10.1016/S0140-6736(03)13693-8
5. Baraff LJ, Lee SI, Schriger DL. Outcomes of bacterial meningitis in children: a meta-analysis. Pediatr Infect Dis J. 1993;12:389–94. DOI: 10.1097/00006454-199305000-00008
6. Grandgirard D, Leib SL. Strategies to prevent neuronal damage in paediatric bacterial meningitis. Curr Opin Pediatr. 2006;18:112–8. DOI: 10.1097/01.mop.0000193292.09894.b7
7. Peltola H. Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clin Microbiol Rev. 2000;13:302–17. DOI: 10.1128/CMR.13.2.302-317.2000
8. Centers for Disease Control and Prevention. Progress toward elimination of *Haemophilus influenzae* type b invasive disease among infants and children: United States, 1998–2000. MMWR Morb Mortal Wkly Rep. 2002;51:234–9.
9. Melegaro A, Edmunds WJ, Pebody R, Miller E, George R. The current burden of pneumococcal disease in England and Wales. J Infect. 2006;52:37–48. DOI: 10.1016/j.jinf.2005.02.008
10. Pathan N, Faust SN, Levin M. Pathophysiology of meningococcal meningitis and septicaemia. Arch Dis Child. 2003;88:601–7. DOI: 10.1136/adc.88.7.601
11. World Health Organization. Outbreak news. Meningococcal disease, African meningitis belt, epidemic season 2006. Wkly Epidemiol Rec. 2006;81:119–20.
12. Anderson MS, Glode MP, Smith AL. Meningococcal disease. In: Feigin RD, Demler GJ, Cherry JD, Kaplan SL, editors. Textbook of pediatric infectious diseases. 5th ed. Philadelphia: Saunders; 2004. p. 1265–79.
13. Jelfs J, Munro R. Epidemiology of meningococcal disease in Australia. J Paediatr Child Health. 2001;37:S3–6. DOI: 10.1046/j.1440-1754.2001.00680.x
14. Ramsay ME, Andrews N, Kaczmarski EB, Miller E. Efficacy of meningococcal serogroup C conjugate vaccine in teenagers and toddlers in England. Lancet. 2001;357:195–6. DOI: 10.1016/S0140-6736(00)03594-7
15. Snape MD, Pollard AJ. Meningococcal polysaccharide-protein conjugate vaccines. Lancet Infect Dis. 2005;5:21–30. DOI: 10.1016/S1473-3099(04)01251-4
16. Nicolas P, Ait M'barek N, Al-Awaidy S, Al BS, Sulaiman N, Issa M, et al. Pharyngeal carriage of serogroup W135 *Neisseria meningitidis* in Hajjees and their family contacts in Morocco, Oman and Sudan. APMIS. 2005;113:182–6. DOI: 10.1111/j.1600-0463.2005.apm1130305.x
17. Lind I, Berthelsen L. Epidemiology of meningococcal disease in Denmark 1974–1999: contribution of the laboratory surveillance system. Epidemiol Infect. 2005;133:205–15. DOI: 10.1017/S0950268804003413
18. Gray SJ, Trotter CL, Ramsay ME, Guiver M, Fox AJ, Borrow R, et al. Epidemiology of meningococcal disease in England and Wales 1993/94 to 2003/04: contribution and experiences of the Meningococcal Reference Unit. J Med Microbiol. 2006;55:887–96. DOI: 10.1099/jmm.0.46288-0
19. Rosenstein NE, Perkins BA, Stephens DS, Lefkowitz L, Cartter ML, Danila R, et al. The changing epidemiology of meningococcal disease in the United States, 1992–1996. J Infect Dis. 1999;180:1894–901. DOI: 10.1086/315158
20. Taha MK, Achtman M, Alonso JM, Greenwood B, Ramsay M, Fox A, et al. Serogroup W135 meningococcal disease in Hajj pilgrims. Lancet. 2000;356:2159. DOI: 10.1016/S0140-6736(00)03502-9
21. Novelli VM, Lewis RG, Dawood ST. Epidemic group A meningococcal disease in Haj pilgrims [letter]. Lancet. 1987;2:863. DOI: 10.1016/S0140-6736(87)91056-7
22. Al-Mazrou YY, Al-Jeffri MH, Abdalla MN, Elgizouli SA, Mishskas AA. Changes in epidemiological pattern of meningococcal disease in Saudi Arabia. Does it constitute a new challenge for prevention and control? Saudi Med J. 2004;25:1410–3.
23. Bakir M, Yagci A, Ulger N, Akbenlioglu C, Ilki A, Soyletir G. Asymtomatic carriage of *Neisseria meningitidis* and *Neisseria lactamica* in relation to *Streptococcus pneumoniae* and *Haemophilus influenzae* colonization in healthy children: apropos of 1400 children sampled. Eur J Epidemiol. 2001;17:1015–8. DOI: 10.1023/A:1020021109462
24. Doganci L, Baysallar M, Saracli MA, Hascelik G, Pahsa A. *Neisseria meningitidis* W135, Turkey. Emerg Infect Dis. 2004;10:936–7.
25. Kilic A, Urwin R, Li H, Saracli MA, Stratton CW, Tang YW. Clonal spread of serogroup W135 meningococcal disease in Turkey. J Clin Microbiol. 2006;44:222–4. DOI: 10.1128/JCM.44.1.222-224.2006
26. Berkley JA, Versteeg AC, Mwangi I, Lowe BS, Newton CR. Indicators of acute bacterial meningitis in children at a rural Kenyan district hospital. Pediatrics. 2004;114:e713–9. DOI: 10.1542/peds.2004-0007
27. Abdillahi H, Poolman JT. Typing of group-B *Neisseria meningitidis* with monoclonal antibodies in the whole-cell ELISA. J Med Microbiol. 1988;26:177–80.
28. Taha MK. Simultaneous approach for nonculture PCR-based identification and serogroup prediction of *Neisseria meningitidis.* J Clin Microbiol. 2000;38:855–7.
29. Tsolia MN, Theodoridou M, Tzanakaki G, Kalabalikis P, Urani E, Mostrou G, et al. The evolving epidemiology of invasive meningococcal disease: a two-year prospective, population-based study in children in the area of Athens. FEMS Immunol Med Microbiol. 2003;36:87–94. DOI: 10.1016/S0928-8244(03)00083-X
30. Saravolatz LD, Manzor O, VanderVelde N, Pawlak J, Belian B. Broad-range bacterial polymerase chain reaction for early detection of bacterial meningitis. Clin Infect Dis. 2003;36:40–5. DOI: 10.1086/345438
31. Gray LD, Fedorko DP. Laboratory diagnosis of bacterial meningitis. Clin Microbiol Rev. 1992;5:130–45.
32. Tzanakaki G, Tsopanomichalou M, Kesanopoulos K, Matzourani R, Sioumala M, Tabaki A, et al. Simultaneous single-tube PCR assay for the detection of *Neisseria meningitidis, Haemophilus influenzae* type b and *Streptococcus pneumoniae.* Clin Microbiol Infect. 2005;11:386–90. DOI: 10.1111/j.1469-0691.2005.01109.x
33. Luby JP. Infections of the central nervous system. Am J Med Sci. 1992;304:379–91. DOI: 10.1097/00000441-199212000-00010
34. Berkman E, Ozben G. Meningococcic meningitis epidemic in Ankara [in Turkish]. Mikrobiyol Bul. 1982;16:101–6.
35. Coskun S, Yanikyurek S, Agzitemiz M. Incidence of epidemiological meningitis in Aegean region. Turk J Infect. 1990;4:431–5.
36. Gazi H, Surucuoglu S, Ozbakkaloglu B, Akcali S, Ozkutuk N, Degerli K, et al. Oropharyngeal carriage and penicillin resistance of *Neisseria meningitidis* in primary school children in Manisa, Turkey. Ann Acad Med Singapore. 2004;33:758–62.

37. Maiden MC, Stuart JM; UK Meningococcal Carriage Group. Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination. Lancet. 2002;359:1829–31. DOI: 10.1016/S0140-6736(02)08679-8
38. Centers for Disease Control and Prevention. Assessment of risk for meningococcal disease associated with Hajj 2001. MMWR Morb Mortal Wkly Rep. 2001;50:221–2.
39. Mayer LW, Reeves MW, Al-Hamdan N, Sacchi CT, Taha MK, Ajello GW, et al. Outbreak of W135 meningococcal disease in 2000: not emergence of a new W135 strain but clonal expansion within the electrophoretic type-37 complex. J Infect Dis. 2002;185:1596–605. DOI: 10.1086/340414

Address for correspondence: Mehmet Ceyhan, Hacettepe University, Faculty of Medicine, Pediatric Infectious Disease Department, Ankara, 06100, Turkey; email: mceyhan@hacettepe.edu.tr

Wide Distribution of a High-Virulence *Borrelia burgdorferi* Clone in Europe and North America

Wei-Gang Qiu,* John F. Bruno,† William D. McCaig,* Yun Xu,† Ian Livey,‡ Martin E. Schriefer,§ and Benjamin J. Luft†

The A and B clones of *Borrelia burgdorferi* sensu stricto, distinguished by outer surface protein C (*ospC*) gene sequences, are commonly associated with disseminated Lyme disease. To resolve phylogenetic relationships among isolates, we sequenced 68 isolates from Europe and North America at 1 chromosomal locus (16S–23S ribosomal RNA spacer) and 3 plasmid loci (*ospC*, *dbpA*, and BBD14). The *ospC*-A clone appeared to be highly prevalent on both continents, and isolates of this clone were uniform in DNA sequences, which suggests a recent trans-oceanic migration. The genetic homogeneity of *ospC*-A isolates was confirmed by sequences at 6 additional chromosomal housekeeping loci (*gap, alr, glpA, xylB, ackA,* and *tgt*). In contrast, the *ospC*-B group consists of genotypes distinct to each continent, indicating geographic isolation. We conclude that the *ospC*-A clone has dispersed rapidly and widely in the recent past. The spread of the *ospC*-A clone may have contributed, and likely continues to contribute, to the rise of Lyme disease incidence.

Multilocus sequence typing (MLST) is the use of DNA sequences at multiple housekeeping loci to characterize genetic variations of natural populations of a bacterial pathogen (*1*,*2*). MLST studies showed that local populations of a bacterial species typically consist of discrete clusters of multilocus sequence types called "clonal complexes," rather than a multitude of randomly assorted genotypes (*2*). Remaining to be tested are how such factors as natural selection, low recombination rate, and genetic drift due to geographic structuring contribute to the formation and maintenance of these clonal complexes in natural bacterial populations (*3*,*4*). Recently, a multilocus sequence analysis approach was proposed to reconstruct phylogenetic histories of bacterial clonal complexes by using concatenated sequences of housekeeping genes when within-loci and between-loci recombinations are infrequent (*5*).

Lyme disease is a multisystem infection, with inflammatory complications that commonly affect the skin, joints, and central nervous system in humans (*6*). Its causative agent, *Borrelia burgdorferi*, a spirochete that parasitizes vertebrates, is transmitted by hard-bodied ticks throughout the temperate zones of the Northern Hemisphere (*7*). Although humans are accidental hosts of *B. burgdorferi,* Lyme disease is the most common vector-borne disease in the United States with >20,000 annual reported cases, 93% of which occurred in 10 northeastern, mid-Atlantic, and north-central states (*8*). Small mammals such as white-footed mice (*Peromyscus leucopus*) and eastern chipmunks (*Tamias striatus*) serve as the main reservoirs of *B. burgdorferi* (*9*,*10*). In Europe, *B. burgdorferi* is transmitted by *Ixodes ricinus* ticks (*11*) and is carried by a large variety of hosts, including birds and small- to medium-sized mammals (*12*).

B. burgdorferi sensu stricto is the primary pathogen of Lyme disease in the United States and is the only pathogenic genospecies that causes Lyme disease in both North America and Europe. More than 12 distinct outer surface protein C (*ospC*) major sequence types coexist in local *B. burgdorferi* sensu stricto populations in the northeastern United States (*13–15*). Sequence variability at *ospC* is the highest among known genomic loci and is strongly linked to variations at other genome-wide loci, with occasional recombinant genotypes caused by plasmid exchanges (*16–19*).

B. burgdorferi sensu stricto intraspecific clonal complexes may differ in their host specificity and degree of hu-

*Hunter College of the City University of New York, New York, New York, USA; †Stony Brook University, Stony Brook, New York, USA; ‡Baxter Innovations GmbH, Orth/Donau, Austria; and §Centers for Disease Control and Prevention, Fort Collins, Colorado, USA

DOI: 10.3201/eid1407.070880

man pathogenecity. Different clonal complexes may prefer different host species (*9*). A restriction fragment length polymorphism type of intergenic spacer (IGS) sequence (corresponding to the *ospC*-A and -B groups) is associated with hematogenous dissemination in patients with early stage Lyme disease (*20*,*21*). Four *ospC* clonal complexes (A, B, I, and K groups) were found to be more likely than others to cause disseminated Lyme disease (*22*). Also, an association of *ospC* clonal types with invasive disease in humans has been found in other pathogenic genospecies such as *B. afzelii* and *B. garinii* (*23*,*24*). However, additional *ospC* clonal types have been isolated in patients with invasive disease (*14*).

Previous molecular assays found a close relationship and overlapping genotypes between the European and North American populations (*25*–*27*). These authors found greater genetic diversity among American strains than European strains and proposed a North American origin for this genospecies. Although these studies provided the first evidence for recent intercontinental migrations, they left the phylogenetic relationships among clonal complexes unresolved because of the use of either anonymous genome-wide markers (e.g., arbitrarily primed PCR), genes with a high recombination rate (e.g., *ospC*), or sequences at a single locus. A phylogeographic approach with multiple molecular markers provides a more robust inference on population history (*28*). Here we obtained a well-resolved phylogeny of *B. burgdorferi* sensu stricto clonal complexes by using multilocus sequence typing at housekeeping loci as well as loci under adaptive evolution. We found evidence of genetic endemism, recent migration events, and recombinant genomic types. In fact, the highly pathogenic *ospC*-A clone seems to have spread rapidly in recent years to infect a broad range of host species in 2 continents.

Materials and Methods

B. burgdorferi Isolates and DNA Isolation

The *B. burgdorferi* sensu stricto isolates were obtained from clinical and tick specimens and cultures from animals in the United States and Europe and maintained as frozen stocks at –70°C (Table 1). For in vitro propagation, a small amount of frozen culture was scraped from the surface of each sample with a sterile inoculating loop and injected into complete Barbour-Stoenner-Kelly II medium (Sigma-Aldrich Corp., St. Louis, MO, USA). Spirochetes were then cultivated at 34°C. All cultures used in this study had undergone a maximum of 2 in vitro passages after recovery from frozen stock. For isolation of genomic DNA, 10 mL of low-passage log-phase bacteria was harvested by

Table 1. *Borrelia burgdorferi* isolates*

Isolates studied†	*ospC* type‡	Biologic origin	US frequency§	EU frequency
B31, CS1, CS2, CS3, 132a, 132b, IP1, IP2, IP3, Ho, HB1, Lenz, L65, PKa2, HII	A	*Ixodes scapularis*, human	6 (New York)	13 (France, Austria, Germany, Italy, Russia)
N40, 88a, 167bjm, SD91, NP14	E	*I. scapularis*, human	3 (New York)	6 (Hungary)
136b, 163b, 297, CS6, CS9, OEA11	K	*I. scapularis,* human	6 (New England)	1 (Hungary)
109a, 160b, 64b, CS7, MI415¶	B1	*I. scapularis,* human, *Peromyscus leucopus*	5 (New York, Michigan)	0
JD1	C	*I. scapularis*	1 (Massachusetts)	0
121a	D	Human	1 (New York)	0
MI407	F	*P. leucopus*	1 (Michigan)	0
72a	G	Human	1 (New York)	0
156a, 156b, MI403, MI411	H	Human, *Tamias striatus*	4 (New York, Michigan)	0
86b, 97b, MI409¶	I	Human, *T. striatus*	3 (New York, Michigan)	0
118a	J	Human	1 (New York)	0
CS8, 80a, MI418¶	N	*I. scapularis,* human, *P. leucopus*	3 (New York, Michigan)	0
94a, CS5	U	Human, *I. scapularis*	2 (New York)	0
Bol12, VS219,¶ Lx36, ZS7	B2	*I. ricinus*, human	0	17 (Finland, Denmark, Switzerland, Italy, Austria, Slovakia, Germany)
Y1, Y10, 217–5, Bol6, Z6	L	*I. ricinus*	0	10 (Finland, Poland, Italy, Austria)
Fr-93/1, Bol15, Bol25, Bol27	Q	*I. ricinus*, *human*	0	4 (Poland, Italy)
Bol26,¶ Z9, PO7	S	*I. ricinus,* human	0	3 (Italy, Austria)
Bol29, Bol30	V	Human	0	15 (Italy, Switzerland, Slovenia, Germany)
SV1	X	*I. ricinus*	0	1 (Finland)
Ri5	W	*I. ricinus*	0	1 (Finland)

**ospC,* outer surface protein C; US, United States; EU, European Union.
†Isolates subjected to multilocus sequence typing analysis.
‡Type names follow (*13*), except that B was split to B1 and B2, and 3 new types (V, X, W) were assigned to European isolates.
§Number and geographic origins of an *ospC* type in our collection.
¶Isolates showing evidence for plasmid-chromosome recombination.

centrifugation at 10,000 rpm for 30 min at 4°C. The bacterial pellet was washed twice with Tris-Cl buffer (10 mmol/L Tris [pH 7.5], 100 mmol/L NaCl), and resuspended in 430 μL TES (10 mmol/L Tris [pH 7.5], 100 mmol/L NaCl, 10 mmol/L EDTA). Subsequently, 10 μL of freshly prepared lysozyme (50 mg/mL), 50 μL Sarkosyl (10%), and 10 μL proteinase K (10 mg/mL) were then added, and the mixture was incubated at 50°C overnight before RNase treatment. After incubation, DNA was extracted with phenol/chloroform and chloroform, precipitated with ethanol, and finally resuspended in TE buffer (1 mmol/L Tris [pH7.5], 1 mmol/L EDTA).

Genomic Markers, PCR Amplifications, and DNA Sequencing

PCR amplifications were attempted at 4 genomic loci for all isolates and at 6 chromosomal housekeeping loci for a genetically representative subset of isolates (Table 2). The IGS locus was chosen for its phylogenetically informative polymorphisms (*16*,*20*). The IGS locus and 6 housekeeping genes (*gap, alr, glpA, xylB, ackA, tgt*) were approximately evenly distributed on the main chromosome based on the B31 genome (*29*). The 3 plasmidborne loci were selected for their high sequence variability and for the absence of close paralogs based on a genome comparison (*17*,*19*). IGS sequences were amplified by using a nested PCR procedure (*30*). Because of high sequence variability, *dbpA* sequences were amplified by using 2 alternative forward primers. PCR amplification was performed in 50 μL containing 200 mmol/L of each dNTP, 2.0 mmol/L $MgSO_4$, 2.5 U of Platinum Taq DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), 0.5 μmol/L of each primer, and 100 ng of genomic DNA template. Following denaturation at 94°C for 1 min, samples underwent 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30s, initial extension at 68°C for 1.5 min, and a final extension step at 68°C for 10 min. PCR products were purified by GFX chromatography (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA), resolved by agarose gel electrophoresis, and visualized by ethidium bromide staining. Purified amplicons were sequenced by using standard dideoxy terminator chemistry as outlined below with the forward and reverse PCR primers. Absence of specific PCR products, indicating potential absence of particular genetic loci or plasmids, was confirmed by follow-up amplifications of the flanking DNA segments.

Automated DNA sequencing of both strands of each fragment was performed by the Stony Brook University Core DNA Sequencing Facility (Stony Brook, NY, USA) by using the dye-terminator method with the same oligonucleotide primers used for PCR amplification or, where required, appropriate internal primers. Sequences were inspected and assembled with the aid of the Sequencher program (Gene Codes, Inc., Ann Arbor, MI, USA). DNA sequences were analyzed by using the BLASTN program through GenBank at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Nucleotide and protein sequence alignments were performed with MacVector version 6.5 (MacVector, Inc., Cary, NC, USA). New sequences were deposited to GenBank under accession nos. EF537321–EF537573.

Phylogenetic Inference and Tests of Population Differentiation

The IGS sequences were used to resolve intraspecific phylogenetic relationships among *B. burgdorferi* isolates (*16*,*20*). Two highly divergent tick isolates from Finland (SV1 and Ri5) were used as outgroups for rooting the phylogenetic tree. IGS sequences were aligned by using ClustalW (*31*). A Bayesian majority-rule consensus tree was estimated by using MrBayes (version 2.1) (*32*) as described previously (*19*). Sequences at the 3 plasmid-borne protein–coding loci were translated into protein sequences and aligned in a pairwise fashion with ClustalW (*31*). Nucleotide alignments were obtained according to the protein alignments. Neighbor-joining trees based on pairwise nu-

Table 2. Genomic markers and PCR primers

Locus*	Primer sequence (5′ → 3′)†	Location‡
BB0057 (*gap*)	F-ATGAAATTGGCTATTAATGG, R-TTGAGCAAGATCAACCACTC	Main chromosome (52.5 K)
BB0160 (*alr*)	F-ATGTATAATAATAAAACAATGG, R-ATTTTCTCTTTTCGTATTTTCC	Main chromosome (160 K)
BB0243 (*glpA*)	F-ATGGAGGAATATTTAAATTTC, R-GTTCATTTTTCCACTCTTC	Main chromosome (249 K)
IGS (*rrs-rrlA*)	1st round§: F-GGTATGTTTAGTGAGGG, R-GGTTAGAGCGCAGGTCTG; 2nd round: F-CGTACTGGAAAGTGCGGCTG, R-GATGTTCAACTCATCCTGGTCCC	Main chromosome (444 K)
BB0545 (*xylB*)	F-ATGAATGCTCTTAGTATTG, R-CCCGTTAACAAATAGAC	Main chromosome (555 K)
BB0622 (*ackA*)	F-TTGTCAAATACAAAAGG, R-AATGTCTTCAAGAATGG	Main chromosome (649 K)
BB0809 (*tgt*)	F-ATGTTTAGTGTAATCAAGAATG, R-ATCGAAATTTTCCTCTTCATAC	Main chromosome (855 K)
BBA24 (*dpbA*)	F1-TAATGTTATGATTAAATG, F2-ATGAATAAATATCAAAAAAC, R-GAAATTCCAAATAACATC	lp54
BBB19 (*ospC*)	F-CCGTTAGTCCAATGGCTCCAG, R-ATGCAAATTAAAGTTAATATC	cp26
BBD14	F-ATGATAATAAAAATAAAAAATAATG, R-ATTTTGATTAATTTTAATTTTGCTG	lp17

*B31 open reading frame (gene) names. IGS, intergenic spacer.
†F, forward; R, reverse.
‡Approximate starting positions on the B31 genome (*29*).
§Source: (*30*).

cleotide sequence distances were inferred by using PHYLIP (*33*) and plotted by using the APE package of the R statistical package (*34*). Genetic differentiation among geographic populations was tested by using the analysis of molecular variance (AMOVA) method implemented in the software package Arlequin 3.1 (*35*). The 6 housekeeping genes were used to infer the overall within- and between-genospecies phylogeny. Sequences of strains B31 and PBi (*B. garinii*) were downloaded from GenBank (*29*,*36*). Sequences of N40, JD1, DN127 (*B. bissettii*), and PKo (*B. afzelii*) were from draft genomes (S. Casjens, pers. comm.). The 6 alignments were concatenated and tested for the presence of gene conversion by using GENECONV with the "within-group fragments only" option (*37*). Two approaches, a Bayesian method with codon site-specific evolutionary rates (using MrBayes) and the other maximum likelihood method with 100 bootstrapped alignments (using DNAML in PHYLIP) (*33*), were used for phylogenetic reconstruction based on concatenated sequences. Branch supports were measured by the posterior probabilities in the Bayesian method and the bootstrap values in the maximum likelihood method.

Results and Discussion

AMOVA Tests of Geographic Differentiation

We sequenced 68 isolates (including 30 from northeastern United States, 6 from the midwestern United States, and 32 from Europe) at a single chromosomal locus (IGS) and 3 plasmid loci (*ospC*, *dbpA*, and BBD14). Using AMOVA, we evaluated the genetic differentiation among geographic samples and found significant genetic differentiation between the North American and European populations at IGS, *ospC*, and *dbpA*, but not BBD14 (Table 3). Among these loci, IGS is the most informative in reflecting the effect of genetic drift caused by geographic isolation because sequence variations at IGS are likely to be selectively neutral. In addition, IGS is on the main chromosome and less likely to be subject to gene conversion. Genetic variations at 3 plasmid loci are more likely to be influenced by natural selection such as adaptation to local vector and host species. Also, plasmid genes are more likely to be transferred so that footprints of geographic isolation might be obscured by gene flow between populations. Natural selection can both enhance and reduce geographic differentiation. With adaptation to local habitats, natural selection acts to enhance the geographic divergence, especially at target loci. On the other hand, diversifying selection within populations inflates within-population diversity, which results in lack of differentiation within populations relative to the within-population polymorphism.

The low level of geographic differentiation at *ospC* showed the divergence-reducing effect of natural selection. Genetic variability of *ospC* is as high within populations as between populations and is caused by diversifying natural selection (*9*,*13*). In such a case, summary statistics such as AMOVA fixation index (F_{ST}) are misleading because sequence cluster analysis showed that most *ospC* alleles have geographically restricted distributions (Figure 1, panel B). The insignificant AMOVA result at BBD14 might be due to a similar effect of high within-population polymorphisms as a result of diversifying selection. In contrast, *dbpA* showed the divergence-enhancing effect of natural selection. The *dbpA* locus showed the highest level of geographic differentiation, owing to a shared allelic type among B2, L, S, Q, and V clonal groups in Europe (Table 3; Figure 1, panel C). An adaptive sweep likely has homogenized these divergent European lineages at *dbpA*.

In summary, on the basis of the neutral genetic variations at IGS, we conclude that the European and North American populations of *B. burgdorferi* sensu stricto have diverged significantly because of genetic drift. Plasmid genes evolved independently and showed various effects of adaptive divergence and diversifying selection. At all 4 loci, genetic variations within the 2 continents contributed to most (>70%) of the total sequence diversity, which suggests recent common ancestry, migration, or both, between the European and North American populations.

Endemic and Shared *ospC* Alleles

Gene trees showed more detailed pictures of geographic variations at each locus (Figure 1). Among the 17 major sequence groups of *ospC*, 2 minor sequence variants

Table 3. Analysis of molecular variance results*†

Locus	Molecular variance, %		Nucleotide diversity, π		Fixation index (F_{ST})‡
	Between continents	Within continents	North America	Europe	
IGS	19.5	80.5	0.0253	0.0243	0.1952§
ospC	3.13	96.87	0.2066	0.1900	0.0313¶
dbpA	26.5	73.5	0.1480	0.0999	0.2650§
BBD14	2.54	97.46	0.0834	0.1333	0.0254 (NS)

*IGS, intergenic spacer; *ospC*, outer surface protein C; NS, not significant (p>0.05).
†Results were obtained by using Arlequin 3.1 (*35*). Samples were 66 IGS sequences divided into 2 continental populations: North America (36 sequences from New York, Connecticut, Massachusetts, and Michigan) and Europe (30 sequences from Italy, Austria, France, Germany, Switzerland, Poland, Hungary, Slovenia, and Finland). Two outgroup sequences (SV1 and Ri5) were excluded from the European sample. Genetic distances between haplotypes were based on the Kimura 2-parameter model.
‡Levels of significance were obtained by 1,000 permutations.
§p<0.001.
¶0.01<p<0.05.

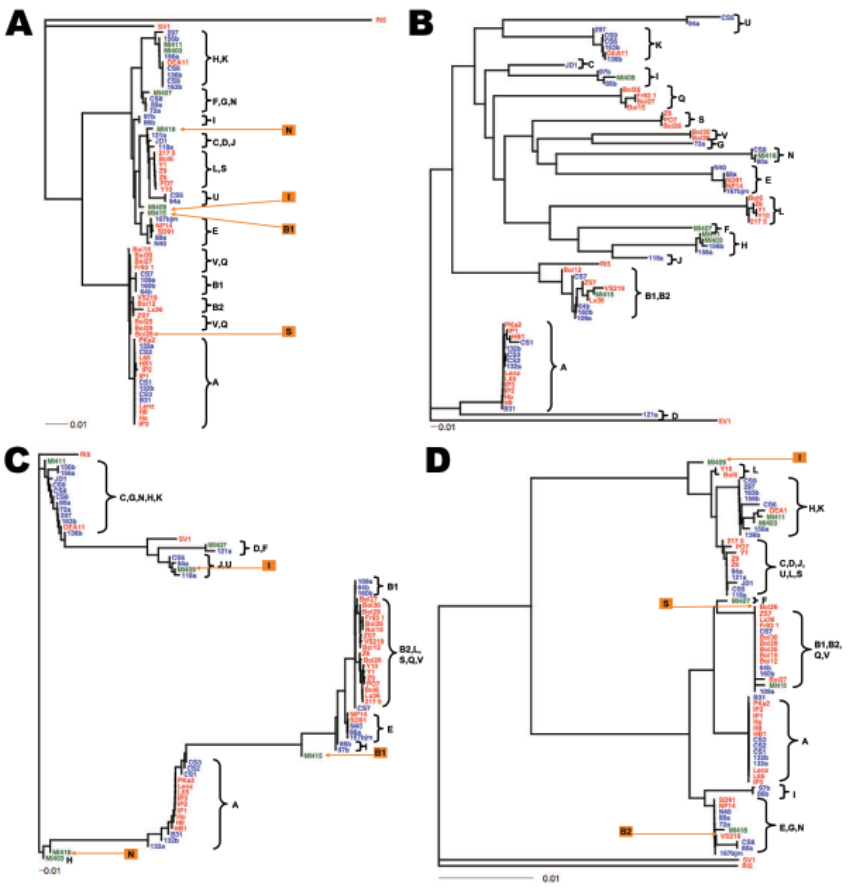


Figure 1. Gene trees showing nucleotide sequence clusters of 68 *Borrelia burgdorferi* isolates at 1 chromosomal locus (panel A: *rrs-rrlA* spacer, or intergenic spacer [IGS]) and 3 plasmid loci (panels B, C, and D: *ospC* on cp26, *dbpA* on lp54, and BBD14 on lp17, respectively). Trees were inferred based on nucleotide sequence alignments and were rooted by using the Ri5, SV1, or both, sequences as outgroups. The DNADIST and neighbor-joining programs of the PHYLIP package (*33*) were used for distance calculation and the APE software package (*34*) was used for tree plotting. Isolates were grouped as clonal groups (A through U), which are named by their typical *ospC* alleles. Five isolates (Bol26, VS219, MI409, MI415, and MI418) showing atypical allelic associations with *ospC* alleles, likely caused by recombination, were labeled in orange. Red, European isolates; blue, northeastern US isolates; green, midwestern US isolates. Scale bars indicate the number of nucleotide substitutions per site.

of major-group allele B were geographically distinct and thereby named B1 in North America and B2 in Europe. Three *ospC* alleles (A, E, and K) were observed in both continents, 5 (B2, S, L, Q, and V) exclusively in Europe (not including the outgroup Ri5 and SV1 alleles), and 10 (B1, C, D, F, G, H, I, J, N, and U) exclusively in North America (Table 1). Although the sample sizes of the North America isolates were small, the same set of *ospC* alleles has repeatedly been identified in surveys of natural populations (*14–16*,*38*). These isolates are therefore a reasonably complete representation of *ospC* diversity in North America. How well our European samples represent the overall *ospC* diversity in Europe is less certain because the European isolates were from an archived collection rather than from systematic surveys of natural populations. For instance, *ospC* alleles J, P, and R have been identified in Europe (*26*). Nonetheless, *ospC*-A appeared to be the only allele that is highly prevalent on both continents (Table 1). An earlier study showed that *ospC*-A and *ospC*-B alleles existed in both continents, whereas other *ospC* alleles were geographically distinct (K, J, F in North America and P, Q, R, S in Europe) (*24*). Our results further suggested that the *ospC*-B clonal group had 2 geographically distinct subtypes (Figure 1, panel B).

Recombinant Genotypes

Previous MLST studies showed complete linkage between *ospC* and other loci on plasmids or the main chromosome in the North American populations (*15*,*16*). This finding is consistent with our study, in which allelic types at IGS, *dbpA*, and BBD14 of the 68 isolates were almost entirely predictable from their *ospC* types. Because of the nearly complete linkage between *ospC* and a locus, individual clonal complexes could conveniently be named after their *ospC* alleles. However, 5 isolates showed alleles at non-*ospC* loci inconsistent with allelic types typically associated with their *ospC* alleles, including MI409, MI415, and MI418 from the midwestern United States and Bol26 and VS219 from Europe (Figure 1). Because these genotypes were new combinations of allelic types found elsewhere, they are more likely to be recombinant genotypes caused by plasmid exchanges, rather than locally evolved new genotypes (*17*). Notably, these probable recombinants were from samples from either the midwestern United States or Europe, and none were from the intensively surveyed northeastern United States. A higher number of clones in the northeastern United States than elsewhere could be understood because *B. burgdorferi* populations in that region are evolutionarily young and show an epidemic

population structure (*15*,*19*). On the basis of the presence of allele types at 4 loci, we determined preliminarily that Bol26 is a group Q or V clone with a transferred *ospC*-S allele because Bol26 clustered with group Q and V isolates at IGS, *dbpA*, and BBD14 (Figure 1). By the same reasoning, VS219 is a group B2 clone with a transferred BBD14 allele. We are currently investigating the donor and recipient genomic types of these recombinant isolates by sequencing 6 additional loci.

Recent Trans-Oceanic Dispersals

Three clonal complexes (A, E, K) are distributed in both continents (Table 1). For the A clonal group, 6 isolates from the United States and 11 isolates from Europe were sequenced at 4 loci. The 4-locus sequences of the isolates between the 2 continents were identical (Figure 1). Thus, the A clonal complex likely was dispersed across the Atlantic Ocean rather recently. To verify the genetic homogeneity of group A isolates from the 2 continents, we randomly selected 4 group A isolates (B31 and 132b from the United States; IP1 and PKa2 from Europe) for further sequencing at an additional 6 chromosomal loci. No fixed sequence differences between 2 continental samples were found, which lends further support for the recent trans-oceanic migration of the A clone (Figure 2). Similarly, the 4-loci sequences of E and K isolates between the 2 continental samples were identical, indicating recent migration of these clonal groups as well (Figure 1). However, the E and K groups seemed less prevalent in Europe than the A group (Table 1). Because individual ticks and hosts are commonly infected with multiple *B. burgdorferi* clones, any migration, whether by natural or human-facilitated mechanisms, is likely to involve a mixture of clonal groups, rather than a single clone. Upon their arrival, however, clonal groups may differ in their ability to colonize a new niche consisting of novel vector and host species. By this reasoning, the A clone is the most ecologically successful strain, able to thrive in a new niche with little genetic change. This conclusion is supported by surveys that showed a broad range of host species for this clonal group (*9*,*10*).

We could not determine conclusively the direction, timing, or number of the trans-oceanic dispersals. Assuming that the chromosomal gene tree in Figure 2, panel B, is an accurate representation of the phylogeny of these clonal groups, a parsimonious scenario is that an early migrant from Europe was the ancestor of the North American clade consisting of the A and B1 groups, and a more recent migration has introduced the A group to Europe. However, none of the basal branches of this gene tree was well supported (Figure 2). Multilocus sequencing of more loci, especially rapidly evolving plasmid loci, of group A isolates will help find more conclusive answers to these questions. To estimate the time of the A clone migration, we noted that no fixed differences in nucleotides occurred within a total of 11,167 aligned bases at 7 chromosomal and 3 plasmid loci. If one assumes a neutral evolutionary rate on the order of 1 substitution per site per million years, the

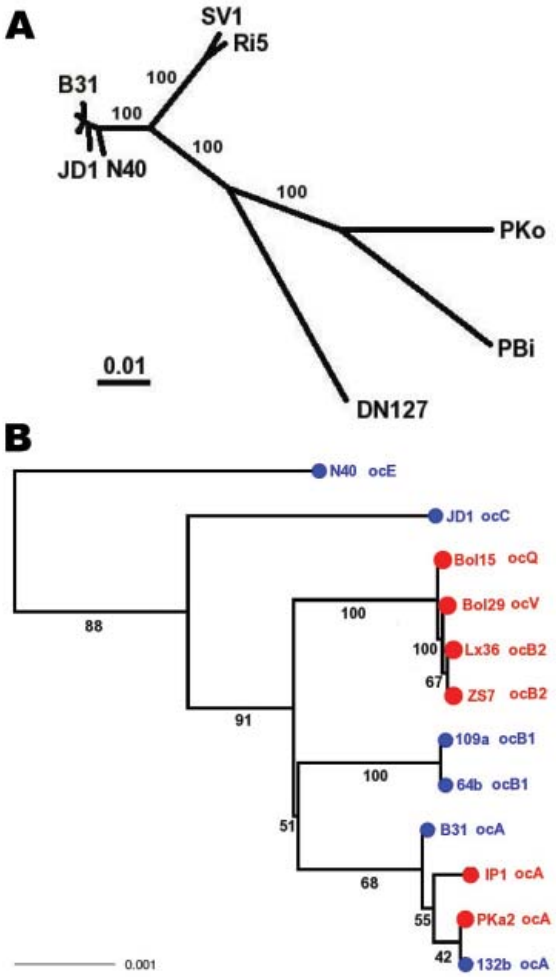


Figure 2. Species phylogeny based on concatenated sequences at housekeeping loci. Seventeen isolates include 1 *Borrelia garinii* strain (PBi), 1 *B. afzelii* strain (PKo), 1 *B. bissettii* strain (DN127), 2 strains of an unnamed genomic species (SV1 and Ri5), and 12 *B. burgdorferi* sensu stricto isolates. These strains were selected for reconstructing interspecies phylogeny (hence species samples), as well as for resolving the clade containing clonal groups A and B (A, B1, and B2 are represented by 2 isolates). Sequences at 6 chromosomal housekeeping loci (*gap, alr, glpA, xylB, ackA,* and *tgt*) were obtained for each strain, with B31 and PBi sequences from published genomes (*29*,*36*), N40, JD1, PKo, and DN127 sequences from draft genomes (S. Casjens et al. pers. comm.). Sequences of the remaining strains were obtained by direct sequencing. The total length of concatenated alignment is 7,509 nt. A) Consensus of maximum likelihood trees obtained by using DNAML of the PHYLIP package (*33*). Branch support values are based on 100 bootstrapped replicates of the original alignment. B) Enlarged view of *B. burgdorferi* sensu stricto subtree. Tips were colored by geographic origin of the isolate (blue, North America; red, Europe) and were labeled with *ospC* major-group allele type. Scale bars indicate the number of nucleotide substitutions per site.

Poisson zero-term probabilities that no fixed difference has occurred within 11,167 bases in the past 50, 100, and 200 years are 0.33, 0.10, and 0.011, respectively. Therefore, the trans-oceanic migration of clone A likely occurred more recently than 200 years ago. More realistic estimates would depend on studies of the neutral mutation rate and generation time of *B. burgdorferi* in the wild.

Phylogenetic Heterogeneity of Group B Isolates

The *ospC*-B clonal group is another highly virulent strain identified by association studies (*20*–*22*,*24*). Initially, group B seemed to be another clone that is distributed in both continents with a few sequence differences at IGS and *ospC* (Figure 1). Sequencing at additional 6 housekeeping loci, however, showed deep phylogenetic heterogeneity of the B group, while the A group remained homogeneous (Figure 2). The 2 B clonal complexes (B1 in North America and B2 in Europe) do not form a monophyletic clade (Figure 2). Rather, B2 clusters with other European clones (V and Q). Also, clones B1 and A, the 2 closest North American relatives, do not form a well-supported clade (only 51% bootstrap support). Clearly, unlike the A clone, the bicontinental distribution of the B clone is not due to recent migration. Sharing of similar *ospC* B alleles between the 2 continents may be due to stabilizing selection or lateral transfer. Because few synonymous changes have occurred between the B1 and B2 alleles, lateral transfer is a more likely cause.

The B2/Q/V showed as a European clade with nearly uniform chromosomal sequences, although it had highly divergent *ospC* alleles (Figure 2). This evidence, based on chromosome-wide genes, strengthens the conclusions of an earlier study that adaptive, large sequence variations at *ospC* are associated with incipient genome divergence (*19*).

Finally, the overall genospecies phylogeny based on MLST showed that the 2 European isolates (Ri5 and SV1) that we used as outgroups may be a new genospecies (Figure 2). This phylogeny is robust because tests of recombination using GENECONV showed no statistically significant gene conversion within the 6 chromosomal housekeeping loci (*37*). The hypothetical genospecies represented by Ri5 and SV1 is more closely related to *B. burgdorferi* sensu stricto than *B. bissettii* (represented here by DN127) is to *B. burgdorferi* sensu stricto. Thus, the MLST phylogeny suggests a possibility that Europe, rather than North America, may be the origin of *B. burgdorferi* sensu stricto, despite a higher contemporary genetic heterogeneity in North America than in Europe.

Conclusions

To summarize, the present study used 7 chromosomal loci (IGS and 6 housekeeping genes) to reconstruct the intra- and interspecific phylogeographic histories of *B. burgdorferi* sensu stricto. Although the standard MLST scheme based on housekeeping genes enables estimates of recombination and mutation rates as well as intraspecific phylogenies (*2*,*5*), our approach of including plasmidborne loci under positive selection helped identify the selective causes of bacterial lineage divergence. Our results showed significant endemic lineage diversification among regional populations, discovered recombinant genotypes, and strongly indicated migrations between North American and European populations in modern times. The highly pathogenic clonal complex A has a prominent presence in both continents, which suggests its success in finding ecologic niches that enable it to infect a broad range of host and vector species. The same genetic basis of the ecologic invasiveness of the *ospC*-A clone may be underlying its high virulence to humans. The emergence of Lyme disease in North America since the 1970s has been attributed to an increasing overlap of human and *B. burgdorferi* habitats (*39*). On the basis of our evidence of migration events, we propose that the trans-oceanic dispersal and colonization of ecologically highly successful clonal complexes (e.g., the A group) may also have played a substantial role.

Acknowledgment

We acknowledge the *Borrelia* sequencing team of Sherwood R. Casjens, John J. Dunn, Benjamin J. Luft, Claire M. Fraser, Weigang Qiu, and Steven E. Schutzer, working under grants from the Lyme Disease Association and National Institutes of Health (AI37256 and AI49003), for access to unpublished sequence information.

Other supports from this work include grants GM083722-01 (toW.-G.Q.) and RR03037 (to Hunter College) from the National Institutes of Health.

Dr Qiu is an assistant professor in the Department of Biological Sciences at Hunter College and the Biology Department in the Graduate Center of the City University of New York. His research interests include the evolution and population biology of infectious diseases, comparative genomics, and bioinformatics tool development.

References

1. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A. 1998;95:3140–5. DOI: 10.1073/pnas.95.6.3140
2. Feil EJ. Small change: keeping pace with microevolution. Nat Rev Microbiol. 2004;2:483–95. DOI: 10.1038/nrmicro904
3. Cohan FM. Concepts of bacterial biodiversity for the age of genomics. In: Fraser CM, Read TD, Nelson KE, editors. Microbial genomes. Totwa (NJ): Humana Press, Inc.; 2004. p. 175–94.
4. Fraser C, Hanage WP, Spratt BG. Neutral microepidemic evolution of bacterial pathogens. Proc Natl Acad Sci U S A. 2005;102:1968–73. DOI: 10.1073/pnas.0406993102

5. Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, et al. Opinion: re-evaluating prokaryotic species. Nat Rev Microbiol. 2005;3:733–9. DOI: 10.1038/nrmicro1236
6. Steere AC, Coburn J, Glickstein L. The emergence of Lyme disease. J Clin Invest. 2004;113:1093–101.
7. Piesman J, Gern L. Lyme borreliosis in Europe and North America. Parasitology. 2004;129(Suppl):S191–220. DOI: 10.1017/S0031182003004694
8. Centers for Disease Control and Prevention. Lyme disease—United States, 2003–2005. MMWR Morb Mortal Wkly Rep. 2007;56:573–6.
9. Brisson D, Dykhuizen DE. ospC diversity in *Borrelia burgdorferi*: different hosts are different niches. Genetics. 2004;168:713–22. DOI: 10.1534/genetics.104.028738
10. Hanincova K, Kurtenbach K, Diuk-Wasser M, Brei B, Fish D. Epidemic spread of Lyme borreliosis, northeastern United States. Emerg Infect Dis. 2006;12:604–11.
11. Rauter C, Hartung T. Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. Appl Environ Microbiol. 2005;71:7203–16. DOI: 10.1128/AEM.71.11.7203-7216.2005
12. Gern L, Estrada-Pena A, Frandsen F, Gray JS, Jaenson TG, Jongejan F, et al. European reservoir hosts of *Borrelia burgdorferi* sensu lato. Zentralbl Bakteriol. 1998;287:196–204.
13. Wang IN, Dykhuizen DE, Qiu W, Dunn JJ, Bosler EM, Luft BJ. Genetic diversity of *osp*C in a local population of *Borrelia burgdorferi* sensu stricto. Genetics. 1999;151:15–30.
14. Alghaferi MY, Anderson JM, Park J, Auwaerter PG, Aucott JN, Norris DE, et al. *Borrelia burgdorferi osp*C heterogeneity among human and murine isolates from a defined region of northern Maryland and southern Pennsylvania: lack of correlation with invasive and noninvasive genotypes. J Clin Microbiol. 2005;43:1879–84. DOI: 10.1128/JCM.43.4.1879-1884.2005
15. Qiu WG, Dykhuizen DE, Acosta MS, Luft BJ. Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the northeastern United States. Genetics. 2002;160:833–49.
16. 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. DOI: 10.1099/mic.0.26944-0
17. Qiu WG, Schutzer SE, Bruno JF, Attie O, Xu Y, Dunn JJ, et al. Genetic exchange and plasmid transfers in *Borrelia burgdorferi* sensu stricto revealed by three-way genome comparisons and multilocus sequence typing. Proc Natl Acad Sci U S A. 2004;101:14150–5. DOI: 10.1073/pnas.0402745101
18. Stevenson B, Miller JC. Intra- and interbacterial genetic exchange of Lyme disease spirochete *erp* genes generates sequence identity amidst diversity. J Mol Evol. 2003;57:309–24. DOI: 10.1007/s00239-003-2482-x
19. Attie O, Bruno JF, Xu Y, Qiu D, Luft BJ, Qiu WG. Co-evolution of the outer surface protein C gene (*ospC*) and intraspecific lineages of *Borrelia burgdorferi* sensu stricto in the northeastern United States. Infect Genet Evol. 2007;7:1–12. DOI: 10.1016/j.meegid.2006.02.008
20. Wormser GP, Liveris D, Nowakowski J, Nadelman RB, Cavaliere LF, McKenna D, et al. Association of specific subtypes of *Borrelia burgdorferi* with hematogenous dissemination in early Lyme disease. J Infect Dis. 1999;180:720–5. DOI: 10.1086/314922
21. Jones KL, Glickstein LJ, Damle N, Sikand VK, McHugh G, Steere AC. *Borrelia burgdorferi* genetic markers and disseminated disease in patients with early Lyme disease. J Clin Microbiol. 2006;44:4407–13. DOI: 10.1128/JCM.01077-06
22. Seinost G, Dykhuizen DE, Dattwyler RJ, Golde WT, Dunn JJ, Wang IN, et al. Four clones of *Borrelia burgdorferi* sensu stricto cause invasive infection in humans. Infect Immun. 1999;67:3518–24.
23. 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. DOI: 10.1016/S0923-2508(01)01186-X
24. Lagal V, Postic D, Ruzic-Sabljic E, Baranton G. Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness. J Clin Microbiol. 2003;41:5059–65. DOI: 10.1128/JCM.41.11.5059-5065.2003
25. Foretz M, Postic D, Baranton G. Phylogenetic analysis of *Borrelia burgdorferi* sensu stricto by arbitrarily primed PCR and pulsed-field gel electrophoresis. Int J Syst Bacteriol. 1997;47:11–8.
26. Marti Ras N, Postic D, Baranton G. *Borrelia burgdorferi* sensu stricto, a bacterial species "Made in the U.S.A."? Int J Syst Bacteriol. 1997;47:1112–7.
27. Postic D, Ras NM, Lane RS, Humair P, Wittenbrink MM, Baranton G. Common ancestry of *Borrelia burgdorferi* sensu lato strains from North America and Europe. J Clin Microbiol. 1999;37:3010–2.
28. Avise JC. Phylogeography: the history and formation of species. Cambridge (MA): Harvard University Press; 2000.
29. Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, et al. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature. 1997;390:580–6. DOI: 10.1038/37551
30. Liveris D, Varde S, Iyer R, Koenig S, Bittker S, Cooper D, et al. Genetic diversity of *Borrelia burgdorferi* in Lyme disease patients as determined by culture versus direct PCR with clinical specimens. J Clin Microbiol. 1999;37:565–9.
31. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80. DOI: 10.1093/nar/22.22.4673
32. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. 2001;17:754–5. DOI: 10.1093/bioinformatics/17.8.754
33. Felsenstein J. PHYLIP—phylogeny inference package. Cladistics. 1989;5:164–6.
34. Paradis E, Claude J, Strimmer K. APE: analyses of phylogenetics and evolution in R language. Bioinformatics. 2004;20:289–90. DOI: 10.1093/bioinformatics/btg412
35. Excoffier L, Laval G, Schneider S. Arlequin ver 3.0: an integratred software package for population data analysis. Evol Bioinform Online. 2005;1:47–50.
36. Glockner G, Lehmann R, Romualdi A, Pradella S, Schulte-Spechtel U, Schilhabel M, et al. Comparative analysis of the *Borrelia garinii* genome. Nucleic Acids Res. 2004;32:6038–46. DOI: 10.1093/nar/gkh953
37. Sawyer S. Statistical tests for detecting gene conversion. Mol Biol Evol. 1989;6:526–38.
38. Wang G, van Dam AP, Dankert J. Evidence for frequent *OspC* gene transfer between *Borrelia valaisiana* sp. nov. and other Lyme disease spirochetes. FEMS Microbiol Lett. 1999;177:289–96. DOI: 10.1111/j.1574-6968.1999.tb13745.x
39. Barbour AG, Fish D. The biological and social phenomenon of Lyme disease. Science. 1993;260:1610–6. DOI: 10.1126/science.8503006

Address for correspondence: Wei-Gang Qiu, Department of Biological Sciences, Hunter College of the City University of New York, 695 Park Ave, New York, NY 10065, USA; email: weigang@genectr.hunter.cuny.edu

AIDS Patient Death Caused by Novel *Cryptococcus neoformans* × *C. gattii* Hybrid

Marjan Bovers,*[1,2] Ferry Hagen,*†[1] Eiko E. Kuramae,* Hans L. Hoogveld,‡ Françoise Dromer,§ Guy St-Germain,¶ and Teun Boekhout*†

Interspecies hybrids of *Cryptococcus neoformans* and *C. gattii* have only recently been reported. We describe a novel *C. neoformans* × *C. gattii* hybrid strain (serotype AB) that was previously described as *C. gattii* and that caused a lethal infection in an AIDS patient from Canada.

Cryptococcus neoformans and *C. gattii* are pathogenic yeasts that may cause meningoencephalitis. *C. neoformans* primarily infects immunocompromised patients and occurs worldwide, whereas *C. gattii* primarily mainly infects otherwise healthy persons and has been thought to occur in subtropical regions (*1*–*3*). However, the recent outbreak of infection with *C. gattii* on Vancouver Island, British Columbia, Canada (*4*), expansion of this outbreak to mainland Canada and the Pacific Northwest region of the United States (*5*), and identification of *C. gattii* isolates in Europe (*6*) show that *C. gattii* can also occur in temperate climates. Molecular techniques can distinguish 7 haploid genotypic groups within *C. neoformans* and *C. gattii* (*7*–*9*; F. Hagen and T. Boekhout, unpub. data).

Recently, 3 serotype BD *C. neoformans* var. *neoformans* × *C. gattii* hybrids were isolated from 2 HIV-negative patients in the Netherlands (*10*). We describe a novel *C. neoformans* var. *grubii* serotype A × *C. gattii* serotype B hybrid that was isolated from an HIV-positive person.

The Study

Strain CBS10496 was isolated from a 31-year-old AIDS patient from Montreal, Quebec, Canada, who had traveled to Mexico ≈15 months before cryptococcosis was diagnosed. The patient died despite extensive antifungal treatment with ketoconazole and amphotericin B (*11*). CBS10496 has been identified as *C. gattii* serotype B (cited as *C. neoformans* var. *gattii*) (*11*). Reference isolates used in this study are listed in the Table.

The ploidy of CBS10496 was determined by using flow cytometry (*10*) with the sequenced haploid strains CBS8710 and CBS10510 as references. Nuclei were visualized after staining with 4′,6-diamidino-2-phenylindole (*10*). Coloration of colonies grown on canavanine-glycine-bromthymol blue (CGB) medium (*12*) was determined after incubation at 24°C for 6 and 15 days. The serotype of CBS10496 was determined by using the CryptoCheck serotyping kit (Iatron Laboratories, Tokyo, Japan).

Ten colonies of CBS10496 were used for DNA extraction (*10*). DNA of these colonies was used for amplified fragment length polymorphism (AFLP) analysis (*7*). The partial sequence of 6 nuclear regions was determined for reference isolates CBS10488–CBS10490, CBS1622, CBS6992, and the putative hybrid isolate CBS10496. Selected nuclear regions were those for internal transcribed spacer (ITS) region, intergenic spacer region, laccase (*CNLAC1*), 2 RNA polymerase II subunits (*RPB1* and *RPB2*), and translation elongation factor 1α (*TEF1*α) (*9*,*10*). Mating types and serotype were determined as described (*10*,*13*).

DNA content of CBS10496 was compared with that of CBS8710 and CBS10510. The G1 peak of reference strains was located at positions 31.6 (CBS8710) and 31.1 (CBS10510), and the G2 peak was located at positions 65.8 (CBS8710) and 56.4 (CBS10510). The G1 peak of CBS10496 was located at position 57.5, and the G2 peak was located at position 115.7. Thus, the G1 peak of CBS10496 coincided with the G2 peak of the haploid strains (Figure 1, panel A), which indicates that CBS10496 has ≈2× more DNA than haploid strains. We concluded that CBS10496 is diploid or aneuploid. Staining with 4′,6-diamidino-2-phenylindole showed that cells of CBS10496 were monokaryotic (Figure 1, panel B).

Reaction of CBS10496 on CGB medium was negative, which corresponds to *C. neoformans* (*12*). The CryptoCheck serotyping kit serum factors 5 (corresponding to serotype B) and 7 (corresponding to serotype A) agglutinated, which indicated that CBS10496 is a serotype AB strain.

The AFLP fingerprint obtained by analysis of colonies of CBS10496 did not match any of the previously defined AFLP genotypes. The fingerprint of CBS10496 was compared with AFLP fingerprints of reference strains CBS8710 and CBS9172, which are AFLP1/VNI, and E566 and CBS10510, which are AFLP4/VGI. The AFLP finger-

*Centraalbureau voor Schimmelcultures–Fungal Biodiversity Centre, Utrecht, the Netherlands; †University Medical Centre Utrecht, Utrecht; ‡Netherlands Institute of Ecology, Nieuwersluis, the Netherlands; §Institut Pasteur, Paris, France; and ¶Laboratoire Santé Publique Quebec, Sainte-Anne-de-Bellevue, Quebec, Canada

DOI: 10.3201/eid1407.080122

[1]These authors contributed equally to this article.

[2]Current affiliation: Netherlands Commission on Genetic Modification, Bilthoven, the Netherlands.

Table. *Cryptococcus* spp. strains used in this study*

Strain†	Mating type‡/serotype	AFLP/M13 genotype§	Source	Country
CBS9172	aA	AFLP1/VNI	Soil sample from garden of patient with neighboring bird colonies	Italy
CBS8710 (H99)	αA	AFLP1/VNI	Patient with Hodgkin disease	United States
CBS1622	αB	AFLP4/VGI	Tumor	France
CBS6992	αB	AFLP4/VGI	Human	Unknown
E566	aB	AFLP4/VGI	*Eucalyptus camaldulensis*	Australia
CBS10510 (WM276)	αB	AFLP4/VGI	Debris of *E. tereticornis*	Australia
CBS10488 (AMC770616)	αBaD	AFLP8/NA	Human CSF	The Netherlands
CBS10489 (AMC2010404)	αBaD	AFLP8/NA	Human CSF	The Netherlands
CBS10490 (AMC2011225)	αBaD	AFLP8/NA	Human CSF	The Netherlands
CBS10496 (LSPQ#308)	αA–B	AFLP9/NA	Blood of an HIV-positive patient	Canada

*AFLP, amplified fragment length polymorphism; CBS, Fungal Biodiversity Centre; AMC, Netherlands Reference Laboratory for Bacterial Meningitis, Academic Medical Center, Amsterdam, the Netherlands; NA, not applicable; CSF, cerebrospinal fluid.
†Origin and genetic composition of the strains are indicated.
‡ –, mating type unknown.
§AFLP fingerprint genotype (*7*), followed by corresponding M13 PCR fingerprint genotype (*8*).

print of CBS10496 contained fragments characteristic of AFLP1/VNI and AFLP4/VGI (Figure 2), which indicated that genetic material from these 2 genotypes was present in this isolate.

Two alleles representing AFLP1/VNI and AFLP4/VGI were found when fragments of *RPB1*, *RPB2*, *CNLAC1*, and intergenic spacer region of CBS10496 were cloned and sequenced. However, after 30 clones were sequenced, only 1 allele was obtained for *TEF1*α, i.e., AFLP4/VGI, and ITS, i.e., AFLP1/VNI. Our results indicate that genetic material from AFLP1/VNI and AFLP4/VGI was present in CBS10496, although only 1 allele was obtained for *TEF1*α and ITS. All sequences were submitted to GenBank (accession nos. DQ286656–DQ286676 and EF102027–EF102072).

Amplification of CBS10496 in a PCR with the *MAT*α and the *MAT*α serotype A–specific primer pair resulted in an amplicon. When *MAT*a and the *MAT*a serotype A–specific PCRs were conducted, no amplicon was obtained. These findings indicate that CBS10496 has a *MAT*α serotype A background. All reference strains yielded amplicons with the expected primer pairs. In addition, CBS10510, a *MAT*α serotype B strain, was amplified with the *MAT*α specific primer pair, and E566, a *MAT*a serotype B strain, yielded an amplicon with the *MAT*a-specific PCR. These results indicate that a *C. gattii* and a *MAT*α serotype A background are present in CBS10496. Because the mating type of the *C. gattii* background within CBS10496 was unknown, 30 *MAT*α clones of CBS10496 were sequenced to determine whether a *MAT*α serotype B allele could be identified.

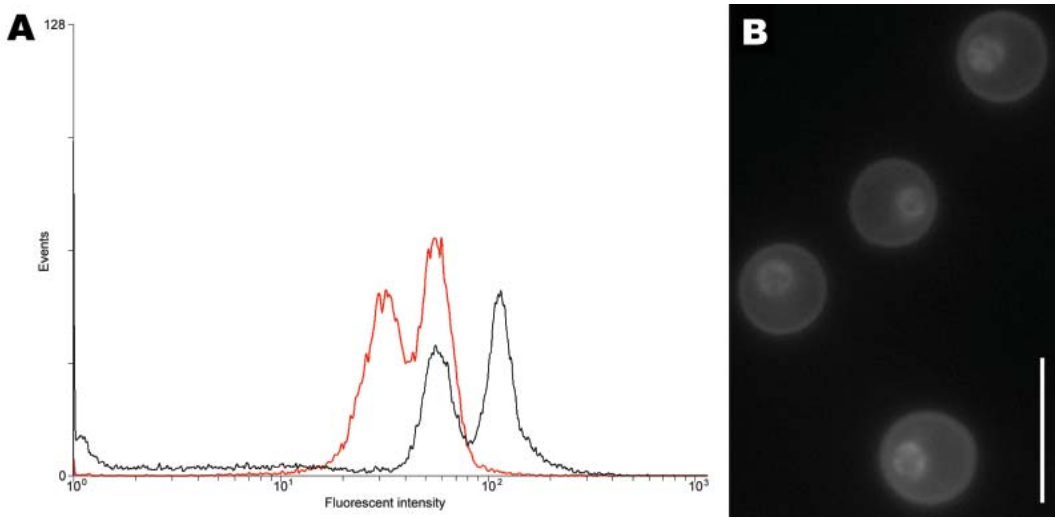


Figure 1. A) Determination of ploidy of the novel *Cryptococcus neoformans* × *C. gattii* serotype AB hybrid isolate CBS10496 by flow cytometry. The first peak corresponds to the G1 phase; the second peak corresponds to the G2 phase. Haploid reference strain CBS10510 is shown by the red line; CBS10496 is shown by the black line. The G1 peak of CBS10496 coincided with the G2 peak of strain CBS10510, which indicated that strain CBS10496 has approximately twice the amount of DNA than CBS10510. B) Nuclear staining of isolate CBS10496 with 4′,6-diamidino-2-phenylindole, showing that cells are monokaryotic. Scale bar = 10 μm.

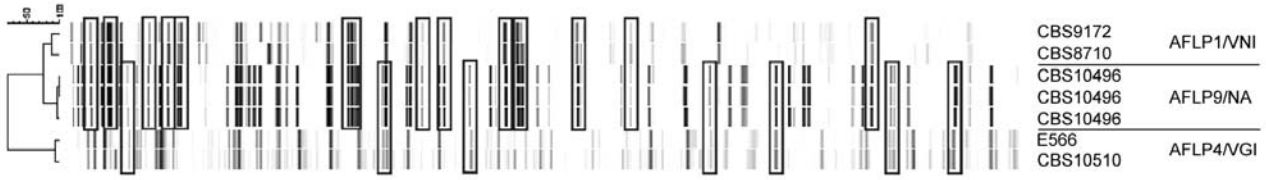


Figure 2. Amplified fragment length polymorphism (AFLP) fingerprint of 3 colonies of the novel *Cryptococcus neoformans* × *C. gattii* hybrid serotype AB isolate CBS10496 and 4 reference strains. CBS9172 and CBS8710 are *C. neoformans* var. *grubii* (AFLP1/VNI) strains; E566 and CBS10510 are *C. gattii* (AFLP4/VGI) strains. Rectangles indicate AFLP fragments characteristic for AFLP1/VNI or AFLP4/VGI and present in isolate CBS10496.

However, all clones were *MAT*α serotype A; no *MAT*α serotype B clones were found.

Conclusions

Our results indicated that CBS10496 is a monokaryotic, diploid, or aneuploid strain with the novel AB serotype. AFLP and sequence analysis showed that the isolate contained fragments of *C. neoformans* var. *grubii* (AFLP1/VNI) and *C. gattii* (AFLP4/VGI). We conclude that this isolate is a novel aneuploid hybrid of *C. neoformans* var. *grubii* (serotype A, AFLP1/VNI) and *C. gattii* (serotype B, AFLP4/VGI).

CBS10496 had been identified as *C. gattii* on the basis of a weak positive reaction on CGB medium (*11*). Our results indicated that CBS10496 was negative on CGB medium. Although a negative response on CGB medium has been shown for other *C. neoformans* × *C. gattii* hybrids (*10*,*14*), weak and delayed positive reactions on CGB medium may occur in *C. neoformans* × *C. gattii* hybrid isolates (*10*,*14*). CBS10496 was previously identified as a serotype B strain (*11*). Inconsistent serotyping results have been reported for other hybrids (*10*,*15*) and may result from differences in specificity and potency among different batches of factor serum. All *C. neoformans* × *C. gattii* hybrids discovered have originated from clinical sources (*13*; F. Hagen and T. Boekhout, unpub. data).

We expected that CBS10496 would have 2 mating-type loci. However, only a serotype A *MAT*α background was observed. Although an amplicon was obtained with *C. gattii*–specific mating-type primers, the *C. gattii* background could not be linked to a mating type. We hypothesize that the serotype AB *C. neoformans* × *C. gattii* hybrid CBS10496 was formed by mating of a *MAT*a serotype B strain with a *MAT*α serotype A strain and subsequent loss of the *MAT*a serotype B allele. Detection of single ITS and *TEF1*α alleles in CBS10496 further supports our findings because it indicates that other alleles were also lost. Loss of genetic material has been observed in other hybrids, such as serotype AD and BD hybrids (*14*), and seems to be a normal process in cryptococcal hybrids.

Our results show that the *C. gattii* parent of the serotype AB hybrid belongs to the AFLP4/VGI genotype, as was the case for serotype BD hybrids (*10*). The *C. gattii* parental sequence of all known serotype BD *C. neoformans* × *C. gattii* hybrid isolates was identical to sequences of AFLP4/VGI strains CBS1622 and CBS6992 in all regions studied (*9*). Detection of 1 specific *C. gattii*–AFLP4/VGI subgroup in all isolated *C. neoformans* × *C. gattii* hybrids may indicate that this subgroup preferentially forms interspecies hybrids.

The research of M.B. and F.H. is supported by the Odo van Vloten Foundation. E.E.K. is supported by the Renewal Fund of the Royal Netherlands Academy of Arts and Sciences.

Dr Bovers participated in this study while conducting doctoral research at the Centraalbureau voor Schimmelcultures–Fungal Biodiversity Centre in Utrecht. She is currently working at the Netherlands Commission on Genetic Modification in Bilthoven. Her research interests include the epidemiology and phylogeny of pathogenic yeasts.

References

1. Speed B, Dunt D. Clinical and host differences between infections with the two varieties of *Cryptococcus neoformans.* Clin Infect Dis. 1995;21:28–34.
2. Sorrell TC. *Cryptococcus neoformans* variety *gattii.* Med Mycol. 2001;39:155–68. DOI: 10.1080/714031012
3. Kwon-Chung KJ, Bennett JE. Epidemiological differences between the two varieties of *Cryptococcus neoformans.* Am J Epidemiol. 1984;120:123–30.
4. Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, et al. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc Natl Acad Sci U S A. 2004;101:17258–63. DOI: 10.1073/pnas.0402981101
5. MacDougall L, Kidd SE, Galanis E, Mak S, Leslie MJ, Cieslak PR, et al. Spread of *Cryptococcus gattii* in British Columbia, Canada, and detection in the Pacific Northwest, USA. Emerg Infect Dis. 2007;13:42–50.
6. Viviani MA, Cogliati M, Esposto MC, Lemmer K, Tintelnot K, Colom Valiente MF, et al. European Confederation of Medical Mycology (ECMM) Cryptococcosis Working Group. Molecular analysis of 311 *Cryptococcus neoformans* isolates from a 30-month ECMM survey of cryptococcosis in Europe. FEMS Yeast Res. 2006;6:614–9. DOI: 10.1111/j.1567-1364.2006.00081.x

7. Boekhout T, Theelen B, Diaz M, Fell JW, Hop WC, Abeln EC, et al. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans.* Microbiology. 2001;147:891–907.
8. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E. the IberoAmerican Cryptococcal Study Group. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003;9:189–95.
9. Bovers M, Hagen F, Kuramae EE, Boekhout T. Six monophyletic lineages identified within *Cryptococcus neoformans* and *Cryptococcus gattii* by multi-locus sequence typing. Fungal Genet Biol. 2007;45:400–21. DOI: 10.1016/j.fgb.2007.12.004
10. Bovers M, Hagen F, Kuramae EE, Diaz MR, Spanjaard L, Dromer F, et al. Unique hybrids between fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii.* FEMS Yeast Res. 2006;6:599–607. DOI: 10.1111/j.1567-1364.2006.00082.x
11. St-Germain G, Noël G, Kwon-Chung KJ. Disseminated cryptococcosis due to *Cryptococcus neoformans* variety *gattii* in a Canadian patient with AIDS. Eur J Clin Microbiol Infect Dis. 1988;7:587–8. DOI: 10.1007/BF01962626
12. Kwon-Chung KJ, Polacheck I, Bennett JE. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). J Clin Microbiol. 1982;15:535–7.
13. Barreto de Oliveira MT, Boekhout T, Theelen B, Hagen F, Baroni FA, Lazera MS, et al. *Cryptococcus neoformans* shows a remarkable genotypic diversity in Brazil. J Clin Microbiol. 2004;42:1356–9. DOI: 10.1128/JCM.42.3.1356-1359.2004
14. Kwon-Chung KJ, Varma A. Do major species concepts support one, two or more species within *Cryptococcus neoformans?* FEMS Yeast Res. 2006;6:574–87. DOI: 10.1111/j.1567-1364.2006.00088.x
15. Viviani MA, Wen H, Roverselli A, Caldarelli-Stefano R, Cogliati M, Ferrante P, et al. Identification by polymerase chain reaction fingerprinting of *Cryptococcus neoformans* serotype AD. J Med Vet Mycol. 1997;35:355–60. DOI: 10.1080/02681219780001411

Address for correspondence: Ferry Hagen, Centraalbureau voor Schimmelcultures–Fungal Biodiversity Center, Uppsalalaan 8, NL-3584CT Utrecht, the Netherlands; email: f.hagen@cbs.knaw.nl

Swine *Trichinella* Infection and Geographic Information System Tools

Robin Burke,* Penny Masuoka,* and K. Darwin Murrell*

Pastured pigs are vulnerable to *Trichinella spiralis* infection through exposure to wild reservoir hosts. To evaluate the potential impact of the expanding production of pork from pasture-raised pigs, we mapped locations of *T. spiralis* occurrence and pastured-pig farms in the United States. Twenty-eight farms were located within 50 km of previous infection.

The incidence of *Trichinella spiralis* infection in humans and swine has declined markedly in North America over the past 20 years; however, sporadic outbreaks still occur (*1*,*2*). The importance of sylvatic reservoir hosts in the persistence of *T. spiralis* infection risk is well-documented, even in countries that have made substantial gains in controlling the infection in swine (*2–5*); *T. spiralis* infection has been recently demonstrated in foxes in Ireland, where no pig infections had been identified for 30 years, (*6*). The outdoor rearing of pigs is a major risk because of increased exposure to sylvatic and synanthropic hosts (*2–10*). Transmission of *T. spiralis* from infected farm pigs to synanthropic (e.g., rats, cats, raccoons) and local sylvatic animal populations also occurs (*3*,*11*).

Pastured-pig operations in the United States have experienced substantial growth in recent years. The number of pigs reared in organic livestock operations, which by law must pasture pigs for at least some part of the day, rose from 1,724 in 2000 to 10,018 in 2005 (*12*). An even larger number of pigs (>100,000) are now being reared nonorganically on pasture and marketed as "pastured, humane, or free-range" pigs. (See below for source of information.) Because of the sporadic occurrence and distribution of outbreaks, and the lack of routine monitoring, the impact of this increase on the risk for *T. spiralis* infection for pastured farm swine is unknown. We report the use of geographic information system (GIS) methods to locate potential high-risk foci to facilitate targeting of surveillance for domestic pig infections, similar to the recent study identifying areas of risk for fascioliasis (*13*).

*Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA

DOI: 10.3201/eid14.07.071538

The Study

Two *Trichinella* databases (All Hosts and Domestic Pig) were compiled by using literature published over the past 60 years (full list of references provided on request from kdmurrell@comcast.net) and *Trichinella* isolate records from North America, maintained at the International *Trichinella* Reference Center in Rome (www.iss.it/site/Trichinella). The All Hosts database contains records on *T. spiralis* infections in wildlife, including synanthropic species such as rats, cats, skunks, and foxes. The second database, Domestic Pig, contains records on *T. spiralis* from domestic pigs. The sylvatic species *T. nativa* and *T. murrelli*, which occur in North America, are not infective for pigs (*Sus scrof*a). *T. pseudospiralis*, which has low infectivity for pigs, has been reported only from a vulture and from a wild boar in North America, but because of the wide range of the former species (12,000–18,000 ha) and the location of wild boars >150 km from a known pastured-pig operation, we excluded this species from our analysis. When latitude and longitude data on host collection sites were not available, we approximated the locations using the coordinate points of the closest town to the collection site. From the 201 *T. spiralis* records that were collected, 54 were selected for mapping (37 wildlife hosts and 17 domestic pig infections). Other records were eliminated either because of vague descriptions of location or because they could not be confirmed as *T. spiralis* rather than a sylvatic species. The infected sylvatic hosts included black bear (*Ursus americanus*), raccoon (*Procyon lotor*), opossum (*Didelphis virginiana*), feral pig/wild boar (*Sus scrofa*), red fox (*Vulpes vulpes*), gray fox (*Urocyon cinereoargenteus*), feral cat (*Felis catus*), striped skunk (*Mephitis mephitis*), coyote (*Canis latrans*), and mink (*Neovison vison*). With the exception of black bears, these wild animals are potentially synanthropic hosts and transfer *T. spiralis* between the sylvatic and domestic habitats (*2–5*).

A third database was created for US farms that raise organic or nonorganically pastured swine. We obtained these data by searching the Internet using the keywords "pasture," "pork," and "organic" for farms producing and marketing pork through the Internet. The latitude/longitude coordinates from town and state data were determined by using the website www.zipinfo.com/search/zipcode.htm.

The databases were converted into map layers within ArcGIS (Environmental Systems Research Institute, Redlands, CA, USA). A basic political boundaries map served as the base map. The western United States is not shown in Figure 1 because the main areas with frequent reports of wild animal and domestic pig *T. spiralis* infections and a prominent pastured-pig industry are the Northeast/

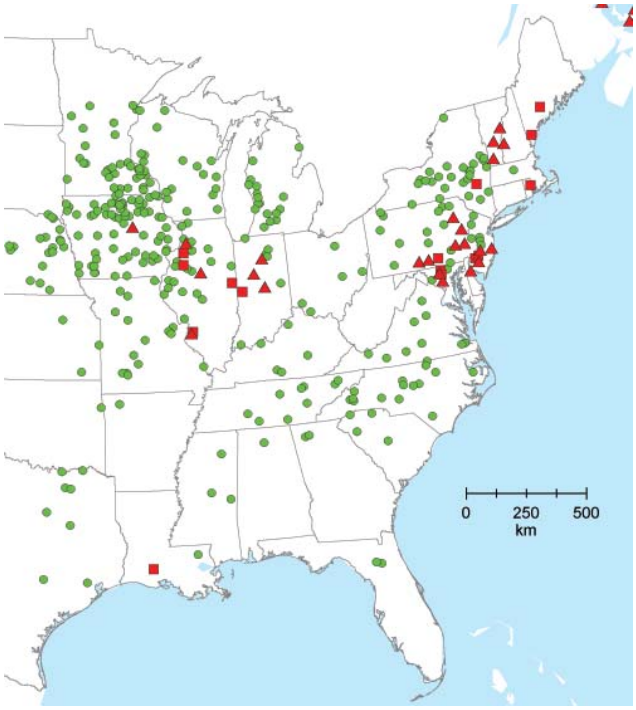


Figure 1. Locations of pastured-pig operations (green dots) and previous records of *Trichinella spiralis* in domestic pigs (red squares) and wildlife (red triangles), United States.

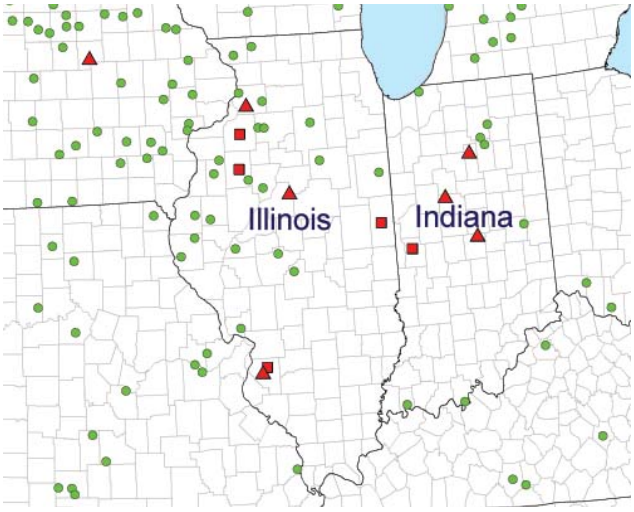


Figure 2. Pastured-pig operations (green dots) and previous records of *Trichinella spiralis* in domestic pigs (red squares) and wildlife (red triangles), Illinois and Indiana.

Middle Atlantic and the central Midwest. Figure 2 shows an enlargement of the Midwest area to illustrate the ability to more precisely locate risk locations (county level). A GIS analysis, using a program in ArcGIS, was performed to measure the distance between pastured-pig farms and historical occurrences of *T. spiralis* in domestic pigs and wildlife. The program calculates a distance between each pastured-pig farm and the nearest *T. spiralis* point on the map and was run 3 times using the following variables: 1) *T. spiralis* in domestic pigs, 2) *T. spiralis* in wildlife, and 3) *T. spiralis* in both pigs and wildlife (Table).

Of the 332 pastured-pig farms mapped, 28 are located within 50 km of documented *T. spiralis* in domestic pigs or wildlife; 6 of these farms are within 50 km of locations with both pig and sylvatic *T. spiralis* infections. An additional 48 pastured-pig operations are within 100 km of *T. spiralis* infection locations.

Conclusions

Using GIS methods to analyze the risk for *T. spiralis* infection associated with the expansion of pastured-pig production, we identified farms that may be at high risk for the introduction of infection into pigs from reservoir hosts. We base this on the fact that the transmission of *T. spiralis* into sylvatic hosts from infected farms can lead to persistence in reservoir hosts (*2*,*3*) and remain a long-term threat to domestic pigs exposed to such hosts in a pasture/dry lot environment (*2*–*11*). The number of pastured-pig farms and records of *T. spiralis* infections are highest in the Northeast and Midwest. Figure 2 demonstrates the ability through map enlargement to identify associations at the local level. In Illinois and Indiana, at least 10 farms within 50 km of previous *T. spiralis* infection in pigs or sylvatic hosts could be identified at the county level. The distances between pastured farms and the locations with recorded foci of *T. spiralis* in wild animals or domestic pigs used in the analysis (Table) are based on the general home ranges for the host species (*14*). For example, raccoons may range up to 3–10 km^2, red foxes 2–10 km^2 (with male dispersal up to 80 km^2), and coyotes up to 50–70 km^2.

These findings should increase the awareness of pastured-pig producers and state veterinary and public health agencies of this potential problem. Targeted surveillance and management prevention programs need to be established in high-risk areas. The use of GIS tools could also help researchers to conveniently locate transmission foci to investigate the measures needed to prevent infection of outdoor-reared pigs. The database we created on pastured-pig

Table. Distances of current pastured-pig operations to locations with past occurrences of *Trichinella spiralis* in domestic pigs or wildlife, United States

Distance to *T. spiralis* infection site and farms, km	Farms near locations of *T. spiralis* in domestic pigs	Farms near locations of *T. spiralis* in wildlife	Farms near locations of *T. spiralis* in both pigs and wildlife	Total
19–50	6	16	6	28
51–100	7	21	20	48

operations is undoubtedly an underestimate of risk because of a lack of a national centralized reporting system for these rearing systems. Furthermore, the infection records are not from a national prevalence survey, which is lacking, but were complied from publications of local surveys and outbreaks (convenience samples). The bias from this method does not, we believe, detract from the objective to introduce the use of GIS tools for identifying foci with potential for *T. spiralis* transmission in outdoor pig-rearing systems. Identification of such foci would provide the opportunity to investigate transmission among wild animals and pigs in agro-ecosystems and the variables that influence transmission, such as climate, pig farm size, herd size, and pig exposure.

This work was carried out in the Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences.

At the time of this study, Dr Burke was a student in the Master's in Public Health program at the Uniformed Services University of the Health Sciences. Currently, she is a laboratory animal medicine resident at the US Army Medical Research Institute for Infectious Diseases at Ft. Detrick, Maryland.

References

1. Roy SL, Lopez AS, Schantz PM. Trichinellosis surveillance—United States, 1997. MMWR Surveill Summ. 2003;52:1–8.
2. Pozio E, Murrell KD. Systematics and epidemiology of *Trichinella.* Adv Parasitol. 2006;63:367–439. DOI: 10.1016/S0065-308X(06)63005-4
3. Murrell KD, Stringfellow F, Dame JB, Leiby DA, Duffy C, Schad GA. *Trichinella spiralis* in an agricultural ecosystem. II. Evidence for natural transmission of *Trichinella spiralis spiralis* from domestic swine to wildlife. J Parasitol. 1987;73:103–9. DOI: 10.2307/3282352
4. Worley DE, Seesee FM, Zarlenga DS, Murrell KD. Attempts to eradicate trichinellosis from a wild boar population in a private game park (USA). In: Campbell WC, Pozio E, Bruschi F, editors. Trichinellosis. Proceedings of the Eighth International Conference on Trichinellosis. Ovieto, Italy. Rome: Instituto Superiore di Sanita Press; 1994. p. 611–6.
5. Doby PB, Murrell KD. Illinois Trichinellosis Control Program. In: Tanner CE, Martinez-Fernandez AR, Bolas-Fernandez F, editors, Trichinellosis. Madrid: Consejo Superior de Investigationes Cientificas Press; 1989. p. 432–8.
6. Rafter P, Marucci G, Brangan P, Pozio E. Rediscovery of *Trichinella spiralis* in red foxes (*Vulpes vulpes*) in Ireland after 30 years of oblivion. J Infect. 2005;50:61–5. DOI: 10.1016/j.jinf.2004.02.004
7. Gamble HR, Boireau P, Kockler K, Kapel CMO. Prevention of *Trichinella* infection in the domestic pig. In: Dupouy-Camet J, Murrell KD, editors. FAO/WHO/OIE guidelines for the surveillance, management, prevention and control of trichinellosis. Paris: World Organisation for Animal Health; 2007. p. 99.
8. Gamble HR, Brady RC, Bulaga LL, Berthoud CL, Smith WG, Detweiler LA, et al. Prevalence and risk association for *Trichinella* infection in domestic pigs in the northeastern United States. Vet Parasitol. 1999;82:59–69. DOI: 10.1016/S0304-4017(98)00267-2
9. Nockler K, Kapel CMO. Detection and surveillance for *Trichinella*. In: Dupouy Camet J, Murrell KD, editors. FAO/WHO/OIE guidelines for the surveillance, management, prevention and control of trichinellosis. Paris: World Organisation for Animal Health; 2007. p. 69–97.
10. van der Giessen J, Fonville M, Bouuknegt M, Langelaar M, Vollema A. Seroprevalence of *Trichinella spiralis* and *Toxoplasma gondii* in pigs from different housing systems in The Netherlands. Vet Parasitol. 2007;148:371–4. DOI: 10.1016/j.vetpar.2007.06.009
11. Leiby DA, Duffy CH, Murrell KD, Schad GA. *Trichinella spiralis* in an agricultural ecosystem: transmission in the rat population. J Parasitol. 1990;76:360–4. DOI: 10.2307/3282667
12. United States Department of Agriculture. Economic Research Service (ERS), Data Sets, Organic Production. Table 5: **c**ertified organic livestock. Data on cows, pigs, sheep, chickens and other poultry, by state, 1997 and 2000–2005 [cited 2007 Mar 21]. Available from http://www.ers.usda.gov/Data/Organic/index.htm#tables
13. Tum S, Puotinen ML, Skerrat LF, Chan B, Sothoen S. A geographic information system for mapping the risk of fascioliasis in cattle and buffaloes in Cambodia. Vet Parasitol. 2007;143:364–7. DOI: 10.1016/j.vetpar.2006.08.033
14. Wilson DE, Ruff S. The Smithsonian book of North American mammals. Washington: Smithsonian Institution Press; 1999.

Address for correspondence: K. Darwin Murrell, Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd, Bethesda, MD 20814, USA; email: kdmurrell@comcast.net

Unique Pattern of Enzootic Primate Viruses in Gibraltar Macaques

Gregory A. Engel,* Mark Pizarro,† Eric Shaw,‡ John Cortes,‡ Agustin Fuentes,§ Peter Barry,¶ Nicholas Lerche,¶ Richard Grant,*[1] Douglas Cohn,# and Lisa Jones-Engel*

Because Gibraltar's macaques (*Macaca sylvanus*) have frequent contact with humans, we assayed 79 macaques for antibodies to enzootic primate viruses. All macaques were seronegative for herpesvirus B, simian T-cell lymphotropic virus, simian retrovirus, simian immunodeficiency virus, and rhesus cytomegalovirus. Seroprevalence of simian foamy virus reached 88% among adult animals.

Animal reservoirs are the most likely sources of emerging infectious diseases that threaten human populations (*1*). Infectious agents from nonhuman primates, in particular, have caused a disproportionate number of new human diseases, leading to calls for international surveillance to monitor the human–nonhuman primate interface. This recognition in turn prompts a need for data focusing on areas and contexts in which humans and nonhuman primates come into contact, creating the potential for zoonotic transmission (*2*,*3*).

Over the past decades, several viruses enzootic to nonhuman primates have been described, and a substantial amount of literature is devoted to their biology, genetics, and capacity to cause disease in humans. The most well known among these, in terms of public health, is simian immunodeficiency virus (SIV), the progenitor of HIV. Other nonhuman primate–borne viruses with known or suspected links to human disease are simian T-cell lymphotropic virus (STLV) and herpesvirus B (Cercopithecine herpesvirus 1) (CeHV-1).

Simian foamy virus (SFV) has received increasing attention as recent findings suggest that human contact with Asian macaques in a number of contexts leads to infection with this virus (*2*,*4*). All free-ranging macaque populations studied to date harbor SFV. Limited data on SFV in humans suggest that infection persists over time. In a small study of 14 humans infected with SFV at primate research centers in the United States, 1 of 7 persons followed consistently over time was shown to have asymptomatic, nonprogressive, monoclonal natural killer cell lymphocytosis (*5*); however, no data link SFV infection with human disease. Further research is needed to determine whether SFV causes immunologic or hematologic effects in humans.

Europe's only population of free-ranging nonhuman primates is found in the Upper Rock Nature Reserve in Gibraltar. These monkeys, also known as the Barbary ape, are macaques of the species *Macaca sylvanus*. When Gibraltar's macaque population dwindled dangerously during World War II, the British government introduced *M. sylvanus* from Morocco and possibly Algeria to prevent local extinction. Over the past few decades, protection and provisioning have led to a steady rise in the population, from ≈30–50 to ≈240 currently.

In addition to their status as a unique wildlife heritage, Gibraltar's macaques are a boon for the local economy. On a typical day, taxi drivers and tour guides ferry thousands of visitors to the Upper Rock Reserve and often use food to entice monkeys to sit on the head or shoulders of visitors (Figure). Each year, >700,000 persons visit the reserve. In recent years, burgeoning macaque populations have become a nuisance for some Gibraltarians living on the edges of the nature reserve, leading to selected culling.

Recent surveys of the Gibraltar macaques have described 6 groups ranging in size from 14 to 64 individuals, with some overlap in range (*6*). Intensity and pattern of contact with humans vary by group; only 3 groups enter into areas visited regularly by tourists. In spite of extensive contact, aggressive interactions with humans are observed less frequently (on average, 170 persons/year are treated for monkey-related injuries) in Gibraltar than in comparable contexts in Asia (*7*). Close human–macaque contact has the potential to transmit infectious agents between the species (*7*,*8*). Given the large amount of interspecies contact occurring in Gibraltar, we sought to characterize the macaques' potential as a reservoir for zoonotic disease.

The Study

We began an ongoing longitudinal serosurvey in 2004, sampling macaques from each of Gibraltar's 6 groups for 6 known enzootic nonhuman primate–borne viruses: SIV, CeHV-1, rhesus cytomegalovirus (RhCMV), STLV, SFV,

*University of Washington National Primate Research Center, Seattle, Washington, USA; †Gibraltar Veterinary Clinic, Gibraltar; ‡Gibraltar Ornithological and Natural History Society, Gibraltar; §University of Notre Dame, Notre Dame, Indiana, USA; ¶University of California, Davis, California, USA; and #Albany Medical College, Albany, New York, USA

DOI: 10.3201/eid1407.071643

[1]Current affiliation: SNBL USA Ltd., Everett, Washington, USA

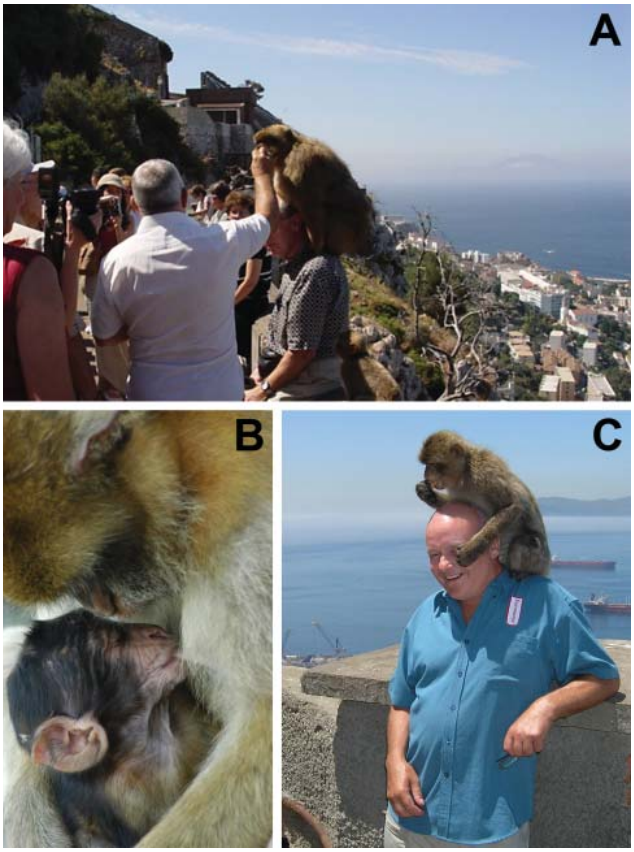


Figure. A) Each year >700,000 tourists visit Gibraltar's Upper Rock Reserve, contributing millions of dollars to the local economy. B) Tourists find Gibraltar' macaques compelling. C) Tour guides use food to entice macaques to perch on visitors, potentially exposing the visitors' mucous membranes to macaque body fluids, a potential route for cross-species transmission of enzootic macaque viruses.

and simian retrovirus (SRV). Trapping, sampling, and assay protocols have been reported elsewhere (*2*,*9*–*11*).

As of 2007, a total of 79 animals were sampled (Table). None of the animals showed evidence of having been infected with SIV, SRV, an alphaherpesvirus antigenically related to CeHV-1, STLV, or with a CMV antigenically related to RhCMV. The complete absence of seroreactivity to CeHV-1 and RhCMV by ELISA was particularly striking because both viruses are considered enzootic in populations of wild-caught and captive macaques (*9*,*13*). In contrast, more than half of the population showed evidence of infection with SFV (Table). Prevalence of SFV infection increased with age, from 20% among young juveniles to ≈90% among adults. No differences in SFV antibody prevalence were detected between male and female macaques or among the 6 groups. Eight macaques were sampled at 2 periods during the study. None of the 5 initially SFV-seronegative macaques had seroconverted by the second sampling. Similarly, all 3 of the initially SFV-seropositive macaques remained seropositive at second sampling.

Conclusions

The Gibraltar macaques are serologically unique. The population shows no evidence of infection with either CeHV-1 or RhCMV, in contrast to other macaque populations studied in which animals seroconvert early in life (*14*). Both these viruses are old viruses that are thought to have coevolved with nonhuman primate species over millions of years. One possible explanation for the lack of CeHV-1 and RhCMV in this population is that the *M. sylvanus* populations in North Africa from which they were derived are not infected with these viruses. At present, the prevalence of these 2 viruses among North African macaques is unknown. If North African *M sylvanus* are infected with CeHV-1 and RhCMV, our results could be explained by a founder effect or the loss of genetic variation when a new colony is established by a very small number of individuals from a larger population. Because seroconversion begins early in life, this hypothesis would imply that the progenitors of the present population were translocated from North Africa when they were young, likely <1 year of age, and therefore less likely to be infected with the viruses.

SFV seroprevalence in Gibraltar macaques mirrors that of other studied macaque populations in South and Southeast Asia with the exception that they seroconvert to SFV at a later age. For example, 81% of free-ranging macaques in Thailand are SFV positive by the age of 3 years, compared with only 38.7% of Gibraltar macaques (*15*). This observation could be explained by decreased rates of aggression because bites are thought to be a principal mode of virus transmission. Properties of the virus or physiologic or immunologic characteristics of the macaques could also influence virus transmission.

The relatively low rates of interspecies aggression observed between macaques and humans in Gibraltar and the low seroprevalence of enzootic nonhuman primate viruses detected in this serosurvey suggest that the risk for virus transmission from macaques to humans in Gibraltar is lower than this risk in other locations where humans come into contact with free-ranging macaques. Nevertheless, the abundance of contact between humans and macaques in Gibraltar implies that the possibility of bidirectional cross-species transmission of infectious agents, including SFV, remains and has implications for humans and macaques. This prospect justifies continued vigilance by local park authorities to monitor and regulate contact between humans and macaques at the Upper Rock Reserve. We recommend strengthened efforts to discourage tourists and locals from feeding macaques and strict enforcement of rules enjoining visitors from seeking contact with them. Macaques should

Table. Seroprevalence of enzootic simian viruses in macaques, Gibraltar, 2004–2007

Category	No. (%)	Seroprevalence, % (no. positive/no. tested)					
		SFV*	SIV†	SRV†	CeHV-1†	STLV†	RhCMV‡
Sex							
Male	45 (57.0)	57.8 (26/45)	0.0	0.0	0.0	0.0	0.0
Female	34 (43.0)	52.9 (18/34)	0.0	0.0	0.0	0.0	0.0
Age class§							
Young juvenile	10 (12.7)	20.0 (2/10)	0.0	0.0	0.0	0.0	0.0
Older juvenile	31 (39.2)	38.7 (12/31)	0.0	0.0	0.0	0.0	0.0
Subadult	13 (16.5)	61.5 (8/13)	0.0	0.0	0.0	0.0	0.0
Adult	25 (31.6)	88.0 (22/25)	0.0	0.0	0.0	0.0	0.0
Total	79	55.7 (44/79)	0.0	0.0	0.0	0.0	0.0

*Simian foamy virus (SFV) seroprevalence for 2004 samples determined using enzyme immunoassay. Subsequent samples screened as part of the whole-virus multiplex flow cytometric assay (*11*).
†Simian immunodeficiency virus (SIV), simian retrovirus (SRV), Cercopithecine herpesvirus 1 (CeHV-1), simian T-cell lymphotropic virus (STLV) seroprevalence for 2004 samples determined by using multiplex microbead assay (*10*); seroprevalence for subsequent samples determined by using whole-virus multiplex flow cytometric assay (*11*).
‡Rhesus cytomegalovirus (RhCMV) seroprevalence for the 2004 samples (n = 40) determined by ELISA according to previously published protocols (*12*). Subsequent samples not screened.
§Age class determinations made on the basis of dental eruption sequence and morphometrics: young juvenile, ≤1 y of age; older juvenile, 1–3 y; subadult, 3–5 y; adult, >5 y.

be fed exclusively in designated areas by trained park personnel; such feeding should compensate for decreased feeding by the general public. Finally, water supplies should be enhanced at feeding sites. These changes will preserve the public's ability to observe the macaques while simultaneously reducing the risk for cross-species transmission of infectious agents.

Acknowledgments

We thank Charles Perez, Dale Lageua, Damian Holmes, and Keith Bensusan, as well as JoAnn Yee, Robin Watanabe, Erik McArthur, and LaRene Kuller for facilitating this research. We also appreciate the 2004 Notre Dame Gibraltar Field School Team, Hanna Engel, and Leah Engel for their help with data collection.

This research has been approved by the University of Washington's International Animal Care and Use Committee (3143-03). Support for this research was provided by National Institutes of Health-National Centers for Research Resources Grant P51 RR000166 and Defense Advance Research Projects Agency Grant N66001-02-C-8072 to LJE. Work in the PB and NL laboratories was supported by the base operating grant of the California National Primate Research Center (RR000169).

Dr Engel is on the faculties of the Swedish Cherry Hill Family Practice Residency and the Department of Family Medicine at the University of Washington. He is a research scientist at the Washington National Primate Research Center. His current research focus is bidirectional pathogen transmission between humans and nonhuman primates in Asia.

References

1. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. Nature. 2008;451:990–3. DOI: 10.1038/nature06536
2. Jones-Engel L, Engel GA, Heidrich J, Chalise M, Poudel N, Viscidi R, et al. Temple monkeys and health implications of commensalism, Kathmandu, Nepal. Emerg Infect Dis. 2006;12:900–6.
3. Jones-Engel L, May CC, Engel GA, Steinkraus KA, Schillaci MA, Fuentes A, et al. Zoonotic transmission of simian foamy viruses in Asia. Emerg Infect Dis. 2008. In press.
4. 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.
5. Boneva RS, Switzer WM, Spira TJ, Bhullar VB, Shanmugam V, Cong ME, et al. Clinical and virological characterization of persistent human infection with simian foamy viruses. AIDS Res Hum Retroviruses. 2007;23:1330–7. DOI: 10.1089/aid.2007.0104
6. Cortes J, Shaw E. The Gibraltar macaques: management and future. In: Hodges JK, Cortes J, editors. The Barbary macaque. Biology, management and conservation. Nottingham (UK): Nottingham University Press; 2006. p. 185-98.
7. Fuentes A. Human culture and monkey behavior: assessing the contexts of potential pathogen transmission between macaques and humans. Am J Primatol. 2006;68:880–96. DOI: 10.1002/ajp.20295
8. Fuentes A, Shaw E, Cortes J. Qualitative assessment of macaque tourist sites in Padangtegal, Bali, Indonesia, and the Upper Rock Nature Reserve, Gibraltar. Int J Primatol. 2007;28:1143–58. DOI: 10.1007/s10764-007-9184-y
9. Huff JL, Eberle R, Capitanio J, Zhou SS, Barry PA. Differential detection of B virus and rhesus cytomegalovirus in rhesus macaques. J Gen Virol. 2003;84:83–92. DOI: 10.1099/vir.0.18808-0
10. Khan IH, Mendoza S, Yee J, Deane M, Venkateswaran K, Zhou SS, et al. Simultaneous detection of antibodies to six nonhuman-primate viruses by multiplex microbead immunoassay. [PMID: 16425999]. Clin Vaccine Immunol. 2006;13:45–52. DOI: 10.1128/CVI.13.1.45-52.2006
11. Kuller L, Watanabe R, Anderson D, Grant R. Development of a whole-virus multiplex flow cytometric assay for antibody screening of a specific pathogen-free primate colony. Diagn Microbiol Infect Dis. 2005;53:185–93. DOI: 10.1016/j.diagmicrobio.2005.05.012
12. Yue Y, Zhou SS, Barry PA. Antibody responses to rhesus cytomegalovirus glycoprotein B in naturally infected rhesus macaques. J Gen Virol. 2003;84:3371–9. DOI: 10.1099/vir.0.19508-0
13. Barry P, Chang W-L. Primate betaherpesviruses. In: Arvin A, Campadielli G, Moore E, Mocarski B, Roizman B, Whitley R, et al., editors. Human herpesviruses: biology, therapy and immunoprophylaxis. Cambridge: Cambridge University Press; 2007. p. 1051–75.

14. Vogel P, Weigler BJ, Kerr H, Hendrickx AG, Barry PA. Seroepidemiologic studies of cytomegalovirus-infection in a breeding population of rhesus macaques. Lab Anim Sci. 1994;44:25–30.
15. Jones-Engel L, Steinkraus KA, Murray SM, Engel GA, Grant R, Aggimarangsee N, et al. Sensitive assays for simian foamy viruses reveal a high prevalence of infection in commensal, free-ranging Asian monkeys. J Virol. 2007;81:7330–7. DOI: 10.1128/JVI.00343-07

Address for correspondence: Lisa Jones-Engel, Division of International Programs, National Primate Research Center, University of Washington, 1705 Pacific St NE, HSB I-039, Seattle, WA 98195, USA; email: jonesengel@bart.rprc.washington.edu

Pneumocystis jirovecii Transmission from Immunocompetent Carriers to Infant

Laura Rivero,*† Carmen de la Horra,*† Marco A. Montes-Cano,*† Alfonso Rodríguez-Herrera,* Nieves Respaldiza,† Vicente Friaza,† Rubén Morilla,† Sonia Gutiérrez,* José M. Varela,*† Francisco J. Medrano,*† and Enrique J. Calderón*†

We report a case of *Pneumocystis jirovecii* transmission from colonized grandparents to their infant granddaughter. Genotyping of *P. jirovecii* showed the same genotypes in samples from the infant and her grandparents. These findings support *P. jirovecii* transmission from immunocompetent carrier adults to a susceptible child.

Pneumocystis jirovecii is an atypical fungus that causes pneumonia in immunosuppressed persons; many questions about its epidemiology and transmission remain unanswered (*1*,*2*). Animal sources for *P. jirovecii* can be excluded because the *Pneumocystis* organisms that infect mammalian species are characterized by strong, close host-species specificity (*3*). Similarly, an environmental reservoir of infection has not been found (*4*). Airborne transmission has been demonstrated in animal models, but the route of transmission of *Pneumocystis* organisms among humans is unclear (*5*). *P. jirovecii* DNA has been identified in ambient air, and airborne transmission between humans is likely (*4*). This hypothesis is supported by reports of case clusters of pneumocystis pneumonia (PcP) among immunosuppressed patients, transmission of *Pneumocystis* DNA from PcP patients to healthcare workers, and transmission of *Pneumocystis* infection from a mother with PcP to her susceptible child (*6*–*10*).

Use of highly sensitive PCR technologies has enabled detection of low levels of *P. jirovecii* in respiratory samples from persons who do not have PcP. Many terms—colonization, carriage, asymptomatic infection, and subclinical infection—have been used to describe these findings. Studies have shown that persons who have underlying HIV infection or other causes of immunosuppression and those who are not immunosuppressed but have chronic lung disease may often be colonized by *P. jirovecii* (*11*–*13*). Further hypotheses claim that these groups may play a role in person-to-person transmission and that they may serve as reservoirs for future *Pneumocystis* infection in other susceptible persons; however, this hypothesis has not been proven.

The Study

A 6-month-old female infant was admitted to Virgen del Rocío University Hospital, Seville, Spain, with a history of nonproductive cough and difficulty breathing. She had been born by vaginal delivery after 40 weeks of gestation, birth weight was 3,490 g, and she had been breast-fed for 2 months. Her mother was healthy and HIV negative. At the time of examination, the infant was afebrile, weighed 4.5 kg (<3rd percentile), and was 62 cm long (<3rd percentile). Respiratory rate was 70 breaths/min; oxygen saturation (by pulse oximetry) was 89%. Fine crackles were heard in both lungs. She had neither lymphadenopathy nor visceromegaly. Diagnostic testing found leukocyte count 12,600 cells/mm^3, CD4+ cells within normal limits, and no immunosupression. Serologic and molecular test results for HIV infection were negative. Chest radiograph showed diffuse interstitial infiltrates suggestive of PcP.

P. jirovecii DNA was detected in nasopharyngeal aspirate samples by amplifying the mitochondrial large-subunit gene of rRNA with nested PCR. No other infections were detected by culture, molecular tests, or serologic tests. The infant was treated with high-dose trimethoprim-sulfamethoxazole and adjuvant steroids. She did well and was discharged a month later.

To determine the origin of the infant's infection, we investigated all persons who lived with her, i.e., parents, brother, and grandparents. Each person underwent clinical and epidemiologic examination and submitted oropharyngeal samples for analysis. Informed consent was obtained from all persons, and the study was approved by the hospital's ethics committee.

The infant's mother, father, and brother were healthy. Her grandmother and grandfather reported a history of rheumatoid arthritis and chronic bronchitis, respectively. None had pneumonia symptoms at the time of the study.

Identification of *P. jirovecii* colonization was carried out by analyzing oropharyngeal samples with nested PCR at the gene encoding the mitochondrial large-subunit rRNA, with primers pAZ102-E and pAZ102-H in the first-round amplification, followed by pAZ102-X and pAZ102-Y in the second-round amplification (*12*). *Pneumocystis* DNA was extracted after samples were digested with proteinase K at 56ºC by using a commercial kit (QIAGEN, Hilden, Germany). To prevent contamination, pipettes with fil-

*Virgen del Rocío University Hospital, Seville, Spain; and †Network Center for Biomedical Research in Epidemiology and Public Health, Seville

DOI: 10.3201/eid1407.071431

ters were used for all manipulations. DNA extraction and preparation of the reaction mixture were performed in 2 different rooms under separate laminar-flow hoods. PCR and analysis of PCR products were performed in another room. Controls were run simultaneously with respiratory samples. Positive controls were bronchoalveolar lavage specimens from PcP patients; negative controls were autoclaved water in the PCR mixture in the absence of the DNA template controls.

All samples that were positive according to nested PCR were sequenced; polymorphisms at nucleotide positions 85 and 248 were detected by direct sequencing (*12*). The nested PCR products were purified by using Sephacryl S-400 columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and reamplified with ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Then for each reaction, 5 μL of PCR product, 4 μL of terminator ready reaction mix, and 3 pmol/L of primer were added. The extension products were purified by ethanol precipitation procedure to remove the excess dye terminators. Each sample pellet was resuspended in 12.5 μL of template suppression reagent and heated at 95ºC for 3 min to denature the product. Electrophoresis was carried out on the ABI prism 310 sequencer (PE Applied Biosystems) in accordance with the manufacturer's recommendations. The sequenced DNA fragments were analyzed by Sequence Navigator version 1.0.1 (PE Applied Biosystems).

P. jirovecii DNA was found in oropharyngeal samples from the infant's grandparents but not her parents or brother. Genotype 1 (85C/248C) was identified in the infant and in her grandparents. Moreover, coinfection with genotype 3 (85T/248C) was detected in the grandfather. In addition, *P. jirovecii* dihydropteroate synthase locus was analyzed in the samples from the infant and her grandparents by PCR restriction fragment-length polymorphism, as described (*12*). Wild dihydropteroate synthase genotype was detected in all samples.

Conclusions

This study provides molecular evidence of *P. jirovecii* transmission from human immunocompetent asymptomatic carriers to a susceptible host, who developed PcP. We cannot exclude the possibility that the cases described were infected by the same environmental source; however, an exosaprophytic form of *P. jirovecii* has not been found (*4*).

P. jirovecii colonization has been shown in pregnant women, and their role as contagious sources for their susceptible newborn infants has been suggested (*14*). In our case, mother-to-infant transmission can be ruled out because the infant's mother was not colonized by *P. jirovecii*. An alternative explanation, but less probable considering the time course of the clinical symptoms, is that the infant acquired the infection in the hospital during delivery and was the source of infection for her grandparents. However, her grandfather was colonized by genotypes 1 and 3, and the infant had only genotype 1.

We hypothesize that the infant was infected by *P. jirovecii* through close contact with her grandparents because they looked after the child full time and lived in the same house. In comparison with animal model experiments on transmission of *Pneumocystis* infection (*5*), the airborne transmission of *P. jirovecii* from the grandfather to the grandmother and the infant is the most probable explanation, especially in view of the high prevalence of *P. jirovecii* colonization of persons with chronic bronchial disease in our area and the grandfather's sputum production associated with this condition (*15*).

This study provides molecular evidence that transmission of *P. jirovecii* from colonized immunocompetent carrier hosts to susceptible persons may occur. The role of persons with chronic pulmonary disease who are colonized with *P. jirovecii* as major reservoirs and sources of infection warrants further investigation.

Acknowledgments

This research is part of the project "*Pneumocystis* Pathogenomics: Unravelling the Colonization-to-Disease Shift," a Coordination Action supported by the European Commission (ERA-NET PathoGenoMics).

This work was partially funded by the Spanish Ministry of Health (FIS 03/1743). C. dlH. and M.A.M.-C. were supported by the Spanish Ministry of Health (FIS CP-04/217 and FIS CM-04/146).

Dr Rivero is a researcher in the Department of Internal Medicine, Virgen del Rocío University Hospital, Seville, Spain. Her main areas of interest are physiopathology and epidemiologic research of human pathogens, such as HIV and *P. jirovecii*.

References

1. Stringer JR, Beard CB, Miller RF, Wakefield AE. A new name (*Pneumocystis jiroveci*) for pneumocystis from humans. Emerg Infect Dis. 2002;8:891–6.
2. Calderón-Sandubete EJ, Varela-Aguilar JM, Medrano-Ortega FJ, Nieto-Guerrer V, Respaldiza-Salas N, de la Horra-Padilla C, et al. Historical perspective on *Pneumocystis carinii* infection. Protist. 2002;153:303–10.et DOI: 10.1078/1434-4610-00107
3. Durand-Joly I, Aliouat EM, Recourt C, Guyot K, Francois N, Wauquier M, et al. *Pneumocystis carinii* f. sp. hominis is not infectious for SCID mice. J Clin Microbiol. 2002;40:1862–5. DOI: 10.1128/JCM.40.5.1862-1865.2002
4. Cushion MT. Pneumocystis: unravelling the cloak of obscurity. Trends Microbiol. 2004;12:243–9. DOI: 10.1016/j.tim.2004.03.005
5. Chabé M, Dei-Cas E, Creusy C, Fleurisse L, Respaldiza N, Camus D, et al. Immunocompetent hosts as a reservoir of *Pneumocystis* organisms: histological and RT-PCR data demonstrate active replication. Eur J Clin Microbiol Infect Dis. 2004;23:89–97. DOI: 10.1007/s10096-003-1092-2

6. Höcker B, Wendt C, Nahimana A, Tönshoff B, Hauser PM. Molecular evidence of *Pneumocystis* transmission in pediatric transplant unit. Emerg Infect Dis. 2005;11:330–2.
7. Rabodonirina M, Vanhems P, Couray-Targe S, Gillibert RP, Ganne C, Nizard N, et al. Molecular evidence of interhuman transmission of *Pneumocystis* pneumonia among renal transplant recipients hospitalized with HIV-infected patients. Emerg Infect Dis. 2004;10:1766–73.
8. Vargas SL, Ponce CA, Gigliotti F, Ulloa AV, Prieto S, Muñoz MP, et al. Transmission of *Pneumocystis carinii* DNA from a patient with *P. carinii* pneumonia to immunocompetent contact health care workers. J Clin Microbiol. 2000;38:1536–8.
9. Miller RF, Ambrose HE, Wakefield AE. *Pneumocystis carinii* f. sp. hominis DNA in immunocompetent health care workers in contact with patients with *P. carinii* pneumonia. J Clin Microbiol. 2001;39:3877–82. DOI: 10.1128/JCM.39.11.3877-3882.2001
10. Miller RF, Ambrose HE, Novelli V, Wakefield AE. Probable mother-to-infant transmission of *Pneumocystis carinii* f. sp. hominis infection. J Clin Microbiol. 2002;40:1555–7. DOI: 10.1128/JCM.40.4.1555-1557.2002
11. Morris A, Kingsley LA, Groner G, Lebedeva IP, Beard CB, Norris KA. Prevalence and clinical predictors of *Pneumocystis* colonization among HIV-infected men. AIDS. 2004;18:793–8. DOI: 10.1097/00002030-200403260-00011
12. Montes-Cano MA, de la Horra C, Martín-Juan J, Varela JM, Torronteras R, Respaldiza N, et al. *Pneumocystis jirovecii* genotypes in Spanish population. Clin Infect Dis. 2004;39:123–8. DOI: 10.1086/421778
13. Vidal S, de la Horra C, Martín J, Montes-Cano MA, Rodríguez E, Respaldiza N, et al. *Pneumocystis jirovecii* colonization in patients with interstitial lung disease. Clin Microbiol Infect. 2006;12:231–5. DOI: 10.1111/j.1469-0691.2005.01337.x
14. Vargas SL, Ponce CA, Sanchez CA, Ulloa AV, Bustamante R, Juarez G. Pregnancy and asymptomatic carriage of *Pneumocystis jiroveci.* Emerg Infect Dis. 2003;9:605–6.
15. Calderón E, de la Horra C, Medrano FJ, López-Suárez A, Montes-Cano MA, Respaldiza N, et al. *Pneumocystis jirovecii* isolates with dihydropteroate synthase mutations in patients with chronic bronchitis. Eur J Clin Microbiol Infect Dis. 2004;23:545–9. DOI: 10.1007/s10096-004-1151-3

Address for correspondence: Enrique J. Calderón, Centros de Investigación Biomédica en Red en Epidemiología y Salud Pública y Servicio de Medicina Interna, Hospital Universitario Virgen del Rocío, Avda, Manuel Siurot s/n, 41013 Seville, Spain; email: sandube@cica.es

Sudden Onset of Pseudotuberculosis in Humans, France, 2004–05

Pascal Vincent,*†‡[1] Alexandre Leclercq,§[1] Liliane Martin,§ *Yersinia* Surveillance Network,§ Jean-Marie Duez,¶ Michel Simonet,*‡[2] and Elisabeth Carniel§[2]

Cases of *Yersinia pseudotuberculosis* infection increased in France during the winter of 2004–05 in the absence of epidemiologic links between patients or strains. This increase represents transient amplification of a pathogen endemic to the area and may be related to increased prevalence of the pathogen in rodent reservoirs.

Yersinia pseudotuberculosis is an enterobacterial pathogen able to grow at low temperatures. It is widespread in the environment (e.g., water, plants), which is the source of contamination for mammals (especially rodents and their predators) and birds (*1*,*2*). Although most cases of human infection are sporadic, outbreaks have occurred in Japan (*3*,*4*), Russia (*5*,*6*), and Finland (*7*,*8*), mainly associated with unchlorinated drinking water or contaminated vegetables. In France, similar increases in case numbers had not been noted until the winter of 2004–05.

The Study

In early January 2005, the French *Yersinia* National Reference Laboratory (YNRL) received 3 *Y. pseudotuberculosis* strains isolated by the same laboratory in Dijon over a 1-week period: 2 from fecal samples of 2 children attending the same daycare center and 1 from the blood of a 65-year-old woman. During the same month, 8 additional strains were isolated from persons in other parts of the country by the *Yersinia* Surveillance Network (based on voluntary participation of 88 hospital-based and private-sector medical laboratories throughout France).

In view of this unusually high number of *Y. pseudotuberculosis* isolations over a short period, the YNRL alerted France's national disease surveillance network, the Institut de Veille Sanitaire, which thereafter performed an epidemiologic investigation. In early February, a request was mailed to all member laboratories in the *Yersinia* Surveillance Network and all 95 microbiology laboratories in university medical centers, asking them to report any recent cases of *Y. pseudotuberculosis* infection. A reminder letter was mailed to all laboratories that had not replied within 1 month of the initial communication. Moreover, from February through April 2005, a total of 76 general medical center laboratories were contacted by telephone and asked to provide the YNRL with any relevant information and/or isolates. Overall, 27 cases of culture-confirmed *Y. pseudotuberculosis* infections were spontaneously reported or actively retrieved (Table 1).

Case reports of *Y. pseudotuberculosis* infection peaked in January 2005. A food-exposure analysis for the first 10 patients did not identify a potential common food source, so a food survey was not performed for subsequent cases. The pseudotuberculosis cases occurred in 19 different counties throughout France, not necessarily the most populated ones (Figure 1). Data on lifestyle and living conditions were obtained for all but 7 patients, of whom 3 had died and 4 (including 2 children) were lost to follow-up. Of the 22 adults, 13 lived in small towns (<5,000 inhabitants) and 5 lived in rural villages (<500 inhabitants). All but 4 lived in houses, as opposed to apartments. Of the 17 adults whose lifestyle was investigated in detail, 14 had a dog, hunted, gardened, and/or grew their own vegetables. In contrast, all 5 children lived in urban areas (>50,000 inhabitants), compared with only 3 of the 22 adults; 4 of the children lived in apartments.

Of the 27 strains isolated, 25 were sent to the YNRL for characterization. All but 4 strains belonged to serotype I, the most common serotype in France. Pulsed-field gel electrophoresis after *Spe*I digestion of genomic DNA showed that (with the exception of the isolates from the 2 children attending the same daycare center) the DNA fingerprints of the 16 other isolates sent to the YNRL during the peak period were all distinct, even when the strains were isolated from the same county (data not shown).

Conclusions

This episode of increased case numbers differs from episodes reported in the literature by the nationwide distribution of cases, the absence of a locally defined cluster, the genetic diversity of the isolates, the predominance of rural residence for patients, and the dominant clinical presentation of septicemia (*3*–*8*). Because the cases were not related to the consumption of a food product sold nationally (e.g., by a supermarket chain), the unknown origin of this phenomenon raises the question of an emerging risk in a new epidemiologic situation.

*Institut National de la Santé et de la Recherche Médicale (INSERM), Lille, France; †Université Lille 2, Lille; ‡Institut Pasteur de Lille, Lille; §Institut Pasteur, Paris, France; and ¶Centre Hospitalier du Bocage, Dijon, France

DOI: 10.3201/eid1407.071339

[1]These authors contributed equally to this article.

[2]These authors contributed equally to the supervision of this work.

Of the 27 patients, 19 lived in a strip of land that stretches from northern France to the Atlantic coast and corresponds to the flyways of small migratory birds. Hence, the avian introduction of strains into the country would have been a possible scenario, as has already been suspected for an epizootic of pseudotuberculosis in an American wildlife park (*9*). Indeed, during the winter of 2004–05, France witnessed a large and unexpected invasion of Bohemian waxwings (*Bombycilla garrulus*) (*10*), a species known to migrate from circumpolar areas where pseudotuberculosis is endemic. At that same time, 3 pseudotuberculosis outbreaks were reported in Siberia (*11*). However, the genetic diversity of the strains isolated from the patients in France and the absence of PCR amplification of the superantigen-encoding gene *ypm* gene (*12*) (which is highly prevalent in Far Eastern strains [*1*]) do not support bird-borne arrival in France of a *Y. pseudotuberculosis* clone from Russia or the Far East.

These cases occurred in areas where other human cases of *Y. pseudotuberculosis* septicemia had been diagnosed (albeit at a much lower rate) in the past 16 years (Figure 2). Exacerbation of a preexisting epidemiologic situation is quite likely. Most previous cases of *Y. pseudotuberculosis* septicemia also concerned inhabitants of low-altitude plains (Figure 2), mainly in rural areas with extensive agriculture zones, which provide favorable habitats for small mammals. Cases were frequently (27.1%) reported in 5 central-western counties of France, where just 4.6% of the population live and incidence of *Francisella tularensis* infection (tularemia) is high (27.3%) (www.invs.sante.fr/surveillance/tularemie/donnees.htm). Moreover, 40 cases of tularemia (with incidence peaks in summer and autumn) were reported to the national surveillance system in 2004 (notification of the disease has been obligatory since 2002), whereas only 8 to 19 cases per year had been reported over the preceding and following periods. Like *Y. pseudotuberculosis*, *F. tularensis* is known to have a rodent reservoir. Hence, the spatial and temporal correlations between human tularemia and pseudotuberculosis in France over re-

Table 1. Relevant characteristics of 27 patients with *Yersinia pseudotuberculosis* infection, France, winter 2004–05*

Age, y	Sex	Risk factors	Main clinical signs/symptoms	Site of organism isolation	O serotype	Illness outcome
0.8	M	None	Diarrhea	Feces	I	Recovery
1	M	None	Diarrhea	Feces	III	Recovery
2	F	None	Diarrhea	Feces	I	Recovery
6	M	None	Diarrhea	Feces	I	Recovery
9	F	None	Diarrhea	Feces	I	Recovery
17	M	Multiple injuries (motorcycle accident)	Postsurgical infection†	Blood	I	Recovery
36	F	HIV infection	Diarrhea, mesenteric adenitis	Feces	I	Recovery
44	F	Bone marrow transplantation	Fever	Blood	I	Death
51	F	Sickle cell anemia, cirrhosis	Diarrhea, esophageal variceal bleeding	Blood	I	Death
59	M	Cirrhosis	Fever, esophageal variceal bleeding	Blood	I	Recovery
61	M	Therapeutic aplasia (colorectal cancer)	Fever, abdominal pain	Blood	I	Death
64	M	Abdominal aortic aneurysm	Abdominal pain	Artery biopsy	I	Recovery
65	F	Myeloma	Fever, septic shock	Blood	I	Death
70	M	Cirrhosis	Fever	Blood	I	Recovery
71	M	Unknown	Abdominal pain	Blood	NA strain	Recovery
71	M	Diabetes, steroid receipt (for retroperitoneal fibrosis)	Fever, diabetes decompensation	Blood	I	Recovery
72	M	Kidney transplantation	Fever	Blood	I	Recovery
74	M	Diabetes	Fever, abdominal pain	Blood	NS	Recovery
75	M	Viral hepatitis C infection	Fever	Blood	I	Recovery
76	M	Cirrhosis	Fever	Blood	NS	Recovery
78	M	Calcific aortic stenosis	Fever, acute heart failure	Blood	I	Recovery
78	M	Diabetes	Fever, septic shock	Blood and artery biopsy	I	Death
79	M	Metastatic colorectal cancer	Fever, respiratory distress syndrome	Blood	I	Death
80	M	Cerebrovascular accident	Fever, cardiogenic shock	Blood	NA strain	Recovery
81	F	Cirrhosis	Fever	Blood	I	Recovery
82	M	Diabetes	Fever, septic shock	Blood	NA strain	Death
83	M	Diabetes	Fever	Blood	I	Recovery

*NA, nonagglutinable; NS, not sent to *Yersinia* National Reference Laboratory.
†This patient's signs/symptoms began after hospitalization; all other patients' signs/symptoms began before hospitalization.

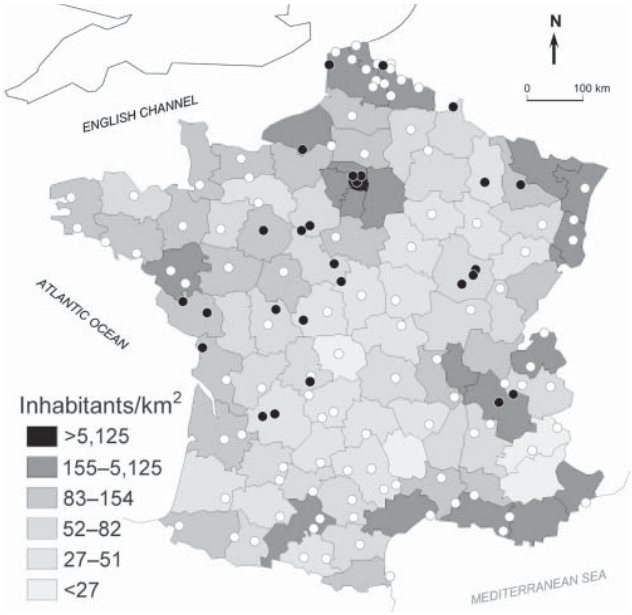


Figure 1. Map of France, showing spatial distribution of *Yersinia pseudotuberculosis* infections during the winter of 2004–05. Black circles, patients' residences; open circles, cities with medical laboratories that stated that they had not isolated any *Y. pseudotuberculosis* from clinical specimens.

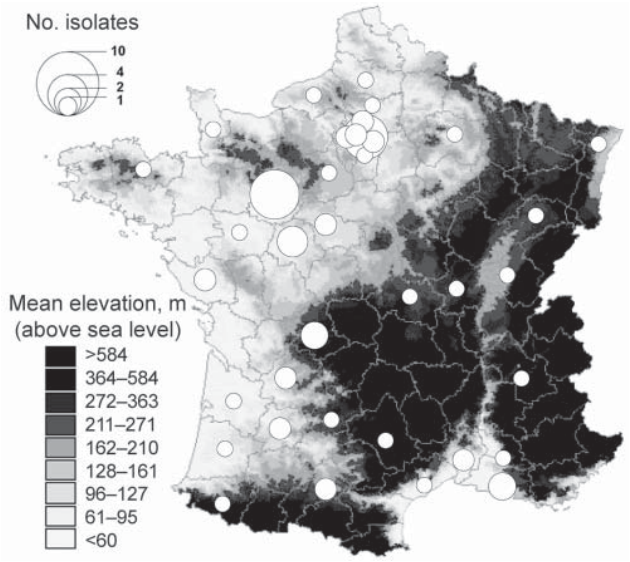


Figure 2. County distribution, France, of *Yersinia pseudotuberculosis* isolated from human blood and reported to the *Yersinia* National Reference Laboratory over the 16 years preceding the winter of 2004–05. The number of isolates is represented by proportionally sized circles arbitrarily located at the center of the counties.

cent years suggest the sudden expansion of a common reservoir in 2004.

Our analysis of the temporal distribution of human *Y. pseudotuberculosis* septicemia cases over the past 16 years showed a peak every 5 years (Table 2). This finding is reminiscent of human hantavirus infections, which have been linked to cyclical oscillations in the vole population (the virus reservoir). Taken as a whole, these data suggest that a rodent reservoir, mainly in rural areas, could have suddenly

Table 2. Temporal distribution of receipt of *Yersinia pseudotuberculosis* blood isolates, France

Period	Monthly isolates*												Annual isolates
	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	
1988–89	0	0	0	0	1	1	2	1	0	0	1	0	6
1989–90	0	0	1	0	1	2	1	0	1	1	0	0	7
1990–91	0	1	0	0	1	1	0	4	0	0	0	0	7
1991–92	0	0	0	1	0	1	1	0	1	0	0	0	4
1992–93	0	0	0	0	1	1	0	1	0	0	0	0	3
1993–94	0	0	0	0	0	0	3	0	2	0	0	0	5
1994–95	0	0	0	0	1	3	1	2	0	0	0	1	8
1995–96	0	0	0	0	1	0	3	0	0	2	0	1	7
1996–97	0	0	0	0	0	1	0	0	0	0	0	0	1
1997–98	0	0	0	0	2	0	0	0	0	0	0	1	3
1998–99	0	0	0	0	0	0	0	1	0	1	0	0	2
1999–00	0	0	0	1	0	2	1	0	0	2	1	1	8
2000–01	0	0	0	0	1	0	0	1	0	2	0	0	4
2001–02	0	0	1	1	0	0	0	0	1	0	0	0	3
2002–03	0	0	0	0	0	1	1	0	0	0	0	0	2
2003–04	0	0	0	0	0	1	4	1	0	2	0	0	8
2004–05†	0	0	0	0	4	4	5	5	0	0	0	0	18
2005–06	0	0	0	0	0	2	2	0	0	0	0	0	4
1988–2006‡	0	1 (0.06)§	2 (0.12)	3 (0.18)	9 (0.53)	16 (0.94)	19 (1.12)	11 (0.65)	5 (0.29)	10 (0.59)	2 (0.12)	4 (0.24)	82 (4.82)

*Received by the French *Yersinia* National Reference Laboratory since September 1988. To encompass the whole cold season, isolates are presented in 12-month periods from September to August.
†Period of increased number of cases.
‡Excludes 2004–05.
§Numbers in parentheses are mean monthly value over the 17 years in which case numbers were not increased.

increased in the spring of 2004, thus increasing the risk for human transmission of *Y. pseudotuberculosis* and *F. tularensis* over the following months.

Rodent populations tend to increase with ongoing changes in agricultural practices, e.g., removal of farmland hedges (which provide shelter for the rodents' predators) and reduction in pesticide use. Hence, the dynamics of the wild rodent population and reduction in pesticide use may represent a useful predictive marker for the occurrence of new outbreaks. The surveillance and control of the small mammal population might help limit the incidence of pseudotuberculosis and other wild rodent–borne diseases of humans in France.

Acknowledgments

We acknowledge the contributions of A. Goudeau, D. Poisson, L. Bret, R. Sanchez, H. Biessy, M. Weber, J.L. Fauchère, G. Chambreuil, P. Geslin, J.M. Seigneurin, G. Grise, J.C. Reveil, C. Auvray, and C. Recule. We also thank H. Porcheret, A. Secher, A. Marmonier, V. Sivadon, and F. Deyroys du Roure for sending the *Y. pseudotuberculosis* isolates to YNRL and/or for providing clinical information about patients. We also acknowledge the help of E. Espié and V. Vaillant for food exposure investigations and that of A. Mailles for obtaining national data on human tularemia.

Dr Vincent, a medical microbiologist at the Lille University Medical Center, is an associate member of the Institut National de la Santé et de la Recherche Médicale Unité 801 research group on the pathogenesis of *Yersinia* species. His main research interests are clinical microbiology and the epidemiology of infectious diseases.

References

1. Fukushima H, Matsuda Y, Seki R, Tsubokura M, Takeda N, Shubin FN, et al. Geographical heterogeneity between Far Eastern and Western countries in prevalence of the virulence plasmid, the superantigen *Yersinia pseudotuberculosis*-derived mitogen, and the high-pathogenicity island among *Yersinia pseudotuberculosis* strains. J Clin Microbiol. 2001;39:3541–7. DOI: 10.1128/JCM.39.10.3541-3547.2001
2. Niskanen T, Waldenstrom J, Fredriksson-Ahomaa M, Olsen B, Korkeala H. virF-positive *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* found in migratory birds in Sweden. Appl Environ Microbiol. 2003;69:4670–5. DOI: 10.1128/AEM.69.8.4670-4675.2003
3. Inoue M, Nakashima H, Ishida T, Tsubokura M. Three outbreaks of *Yersinia pseudotuberculosis* infection. Zentralbl Bakteriol Mikrobiol Hyg [B]. 1988;186:504–11.
4. Nakano T, Kawaguchi H, Nakao K, Maruyama T, Kamiya H, Sakurai M. Two outbreaks of *Yersinia pseudotuberculosis* 5a infection in Japan. Scand J Infect Dis. 1989;21:175–9. DOI: 10.3109/00365548909039966
5. Somov GP, Martinevsky IL. New facts about pseudotuberculosis in the USSR. On the outcome of the first conference on human pseudotuberculosis in the USSR. Contrib Microbiol Immunol. 1973;2:214–6.
6. Smirnova YY, Tebekin AB, Tseneva GY, Rybakova NA, Rybakov DA. Epidemiological features of *Yersinia* infection in a territory with developed agricultural production. Epinorth 2004 [cited 2005 April 1]. Available from http://www.epinorth.org/artikler/?id=44884
7. Jalava K, Hallanvuo S, Nakari UM, Ruutu P, Kela E, Heinasmaki T, et al. Multiple outbreaks of *Yersinia pseudotuberculosis* infections in Finland. J Clin Microbiol. 2004;42:2789–91. DOI: 10.1128/JCM.42.6.2789-2791.2004
8. Hallanvuo S, Nuorti P, Nakari UM, Siitonen A. Molecular epidemiology of the five recent outbreaks of *Yersinia pseudotuberculosis* in Finland. Adv Exp Med Biol. 2003;529:309–12.
9. Welsh RD, Ely RW, Holland RJ. Epizootic of *Yersinia pseudotuberculosis* in a wildlife park. J Am Vet Med Assoc. 1992;201:142–4.
10. Fouarge J, Vandevondele P. Synthesis on the exceptional invasion of waxwings (*Bombycilla garrulus*) in Europe in 2004–2005 [in French]. Aves. 2005;42:281–311.
11. Yersiniosis—Russia (Siberia). Promed. 2005 Apr 27. Available from http://www.promedmail.org, archive no: 20050427.1169.
12. Carnoy C, Floquet S, Marceau M, Sebbane F, Haentjens-Herwegh S, Devalckenaere A, et al. The superantigen gene *ypm* is located in an unstable chromosomal locus of *Yersinia pseudotuberculosis.* J Bacteriol. 2002;184:4489–99. DOI: 10.1128/JB.184.16.4489-4499.2002

Address for correspondence: Pascal Vincent, Service de Bactériologie-Hygiène, Centre de Biologie Pathologie/Centre Hospitalier Régional Universitaire de Lille, bd du Pr J. Leclercq, F-59 037 Lille CEDEX, France; email: p-vincent@chru-lille.fr

Molecular Typing of *Trypanosoma cruzi* Isolates, United States

Dawn M. Roellig,* Emily L. Brown,* Christian Barnabé,† Michel Tibayrenc,† Frank J. Steurer,‡ and Michael J. Yabsley*

Studies have characterized *Trypanosoma cruzi* from parasite-endemic regions. With new human cases, increasing numbers of veterinary cases, and influx of potentially infected immigrants, understanding the ecology of this organism in the United States is imperative. We used a classic typing scheme to determine the lineage of 107 isolates from various hosts.

In Latin America, an estimated 10–12 million persons are infected with *Trypanosoma cruzi*, the etiologic agent of Chagas disease and a major contributor to heart disease within the region. Autochthonous human infections in the United States have been reported in 6 persons, with the most recent case reported from Louisiana (*1*). In addition, the parasite is euryxenous; it is able to infect a broad range of hosts, including domestic dogs, woodrats, raccoons, opossums, armadillos, and nonhuman primates.

Associations between host species and parasite genotype have been suggested and are important in understanding the domestic and sylvatic cycles of *T. cruzi* (*2*–*4*). Although studies conducted on US isolates suggest an association between *T. cruzi* genotype and host, these studies were limited because of low sample numbers, low host diversity, and narrow geographic distribution (*2*,*4*–*7*). In the current investigation, we used the molecular typing scheme proposed by Brisse et al. (*8*), in which isolates are delineated into 1 of the 6 lineages (types I and IIa–IIe) on the basis of size polymorphisms of several PCR markers. We then expanded characterization of US isolates and show additional evidence for correlations between host specificity and genotype of *T. cruzi*.

The Study

We analyzed 107 isolates of *T. cruzi* from multiple species of free-ranging and captive wildlife, domestic animals, triatomine bug vectors, and humans who were autochthonously infected in the United States. Some isolates were obtained as liquid nitrogen–stored parasites from the Centers for Disease Control and Prevention (Atlanta, GA, USA), the Institut Pasteur (Paris, France), and the Southeastern Cooperative Wildlife Disease Study (Athens, GA, USA) and were established in axenic liver infusion tryptose medium as described (*9*). Additional isolates were obtained from wild-trapped animals in axenic liver infusion tryptose medium or canine macrophage cell culture as described (*10*). Isolated DNA was used as template for PCR amplification of 3 gene targets, mini-exon, D7 divergent domain of 24S α rRNA, and 18S rRNA, according to published methods (*8*). Locality data and results of molecular typing of each isolate are shown in the online Appendix Table (available from www.cdc.gov/EID/content/14/7/1123-appT.htm). All animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee and under animal use protocol approved by the Institutional Animal Care and Use Committee at the University of Georgia.

Only 2 genotypes, *T. cruzi* I and *T. cruzi* IIa, were detected. Typical amplicon sizes of *T. cruzi* I and *T. cruzi* IIa isolates from the United States are shown in the Table. Atypical banding patterns and isolates that differ from the standard genotype from a particular host are also represented. With the exception of human isolates, 1 primate isolate, and a few raccoon isolates, placental mammalian isolates, including those from raccoons, domestic dogs, ring-tailed lemurs, and skunks, were characterized as type IIa (online Appendix Table). All remaining isolates, including those from Virginia opossums (*Didelphis virginiana*), triatomine vectors, humans, and rhesus macaques from the United States, were identified as type I (online Appendix Table).

Conclusions

In contrast to studies conducted on South American isolates, for which 6 genotypes of *T. cruzi* have been identified, only 2 genotypes (I and IIa) were identified in the current study. These data support results of investigations in Central America and Mexico in which a paucity of genotypes was found (*14*,*15*). Many investigations on *T. cruzi* evolutionary ecology have shown strict host–parasite specificity in regard to host species and parasite genotype (*2*–*4*), although exceptions have been observed. The presence of only 2 genotypes in the United States could be caused by a lack of introduction of other genotypes or a lower diversity of natural reservoir hosts for *T. cruzi* than in South America. A recent analysis of *T. cruzi* hosts in North and South America indicated that ≥48 host species representing 17 families were infected with ≥1 of the 6 strains (*4*). Only 6 of these hosts have established populations in the United States, and US isolates from these species were only characterized as types I or IIa (*4*).

*University of Georgia, Athens, Georgia, USA; †Institut de Recherche pour le Developpement, Montpellier, France; and ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: 10.3201/eid1407.080175

Our data for US isolates correspond with those of previous studies in which *Didelphis* spp. are reservoirs for type I *T. cruzi* (*4*); no infections with type II parasites were observed. The Virginia opossum (and its ancestors), which is the only marsupial present in the United States (it migrated from South America ≈4.5 million years ago), is a possible host for *T. cruzi* I. This evidence suggests that *T. cruzi* was not recently introduced into North America or the United States (*5*). Additionally, sufficient time may have passed for random and rare genetic exchange events to occur independent of those found in South American isolates (*13*), enabling the lineage to infect atypical reservoirs (i.e., raccoons) in North America.

The second major natural reservoir of *T. cruzi* in the United States is the raccoon. In general, the nonprimate placental mammals in our study were infected with type IIa, a strain that is commonly found in sylvatic cycles in the Southern Cone of South America. Our data confirm previous typing of US isolates by multilocus enzyme electrophoresis or random amplified polymorphic DNA analysis (*5*), in which 11 raccoons from Georgia were characterized as zymodeme 3 (equivalent to IIa). Although raccoons are predominately infected with *T. cruzi* IIa, 4 known exceptions include 3 isolates from Georgia and Florida in the current study and 1 raccoon from Louisiana from a previous study (*5*).These data are in contrast to typing data for Virginia opossum isolates, which have all found *T. cruzi* I. This finding suggests that opossums primarily maintain persistent infections with *T. cruzi* I.

All characterized human isolates from autochthonous US cases of infection with *T. cruzi* are *T. cruzi* I. This genotype is predominantly responsible for Chagas disease north of the Amazon Basin and is part of the domiciliary cycle of the parasite. Our findings correspond with data from Mexico where *T. cruzi* I is the predominate strain detected in humans (*14*). It would be useful to differentiate biologic characteristics and polymorphisms by using additional gene targets in human type I isolates and compare them with those in opossum, triatomine vectors, and rhesus macaque isolates from the United States. Additionally, comparing these US isolates and Mexican reference strains with those from South America may indicate why type I typically infects humans in North America and multiple strains are found in humans in South America.

Our results provide additional evidence that *T. cruzi* has distinct genotypes that preferentially infect 1 host species or a group of hosts. Humans and marsupials are typically infected with type I *T. cruzi*, but raccoons, skunks, domestic dogs, and prosimians are typically infected with type IIa. Although we only detected *T. cruzi* I in triatomid bugs, other studies have detected *T. cruzi* IIa in triatomids from the United States (*5*). The mechanism is unknown by which persistent infections with a particular genotype of *T. cruzi* develop in certain hosts. Further analysis of isolates from an increased host diversity and geographic range should be pursued. Determining basic infection dynamics of reservoir hosts experimentally infected with various *T. cruzi* genotypes may provide additional insight into the host–parasite dichotomy.

Acknowledgments

We thank B. Wilcox, B. Hanson, and D. Kavanaugh for field assistance; C. Paddock for providing 1 isolate used in the study; and P. Dorn for providing blood for isolation of 1 isolate.

This study was supported by grant R15 AI067304 from the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

Ms Roellig is a doctoral student in infectious diseases at the University of Georgia. Her research interests are vector-borne zoonotic diseases, including Chagas disease in wildlife and tick-borne rickettsial pathogens.

References

1. Dorn PL, Perniciaro L, Yabsley MJ, Roellig DM, Balsamo G, Diaz J, et al. Autochthonous transmission of *Trypanosoma cruzi*, Louisiana. Emerg Infect Dis. 2007;13:605–7.
2. Clark CG, Pung OJ. Host specificity of ribosomal DNA variation in sylvatic *Trypanosoma cruzi* from North America. Mol Biochem Parasitol. 1994;66:175–9. DOI: 10.1016/0166-6851(94)90052-3

Table. Approximate amplicon sizes of gene targets and lineage determination in *Trypanosoma cruzi*

Strain	Mini-exon, bp	24S α rRNA, bp	18S rRNA, bp	Lineage
FL Opo 15*	350	110	175	I
GA Rac 103*	None	120	155	IIa
FL Rac 5*	400	120	155	IIa
93053103R cl3	350	110	175	I
FL Rac 13	350	110, 120	155, 175	I/IIa†
FL Rac 46	400	110, 120	155	I/IIa†
Griffin Dog	350	110, 120	155	I/IIa†
Monk RH89–40	None	110	155	I/IIa†

*Denotes isolates used as representative banding patterns seen for classic lineage typing.
†Because of atypical banding patterns, a clear definition of an isolate as type I vs. type IIa could not be obtained.

3. Briones MR, Souto RP, Stolf BS, Zingales B. The evolution of two *Trypanosoma cruzi* subgroups inferred from rRNA genes can be correlated with the interchange of American mammalian faunas in the Cenozoic and has implications to pathogenicity and host specificity. Mol Biochem Parasitol. 1999;104:219–32. DOI: 10.1016/S0166-6851(99)00155-3
4. Yeo M, Acost N, Llewellyn M, Sánchez H, Adamson S, Miles GA, et al. Origins of Chagas disease: *Didelphis* species are natural hosts of *Trypanosoma cruzi* I and armadillos hosts of *Trypanosoma cruzi* II, including hybrids. Int J Parasitol. 2005;35:225–33. DOI: 10.1016/j.ijpara.2004.10.024
5. Barnabé C, Yaeger R, Pung O, Tibayrenc M. *Trypanosoma cruzi*: a considerable phylogenetic divergence indicates that the agent of Chagas disease is indigenous to the native fauna of the United States. Exp Parasitol. 2001;99:73–9. DOI: 10.1006/expr.2001.4651
6. Miles MA, Souza A, Povoa M, Shaw JJ, Lainson E, Toye PJ. Isozymic heterogeneity of *Trypanosoma cruzi* in the first autochthonous patients with Chagas' disease in Amazonian Brazil. Nature. 1978;272:819–21. DOI: 10.1038/272819a0
7. Yabsley MJ, Noblet GP. Biological and molecular characterization of a raccoon isolate of *Trypanosoma cruzi* from South Carolina. J Parasitol. 2002;88:1273–6.
8. Brisse S, Verhoef J, Tibayrenc M. Characterisation of large and small subunit rRNA and mini-exon genes further support the distinction of six *Trypanosoma cruzi* lineages. Int J Parasitol. 2001;31:1218–26. DOI: 10.1016/S0020-7519(01)00238-7
9. Castellani O, Ribeiro LV, Fernandes JF. Differentiation of *Trypanosoma cruzi* in culture. J Protozool. 1967;14:447–51.
10. Yabsley MJ, Norton TM, Powell MR, Davidson WR. Molecular and serologic evidence of tick-borne ehrlichiae in three species of lemurs from St. Catherine's Island, Georgia, USA. J Zoo Wildl Med. 2004;35:503–9.
11. de Freitas JM, Augusto-Pinto L, Pimenta JR, Bastos-Rodrigues L, Goncalves VF, Teixeira SM, et al. Ancestral genomes, sex and the population structure of *Trypanosoma cruzi.* PLoS Pathog. 2006;2:e24. DOI: 10.1371/journal.ppat.0020024
12. Brisse S, Barnabé C, Tibayrenc M. *Trypanosoma cruzi* clonal diversity: identification of discrete phylogenetic lineages by random amplified polymorphic DNA and multilocus enzyme electrophoresis analysis. Int J Parasitol. 2000;30:35–44. DOI: 10.1016/S0020-7519-(99)00168-X
13. Machado CA, Ayala FJ. Nucleotide sequences provide evidence of genetic exchange among distantly related lineages of *Trypanosoma cruzi.* Proc Natl Acad Sci U S A. 2001;98:7396–401. DOI: 10.1073/pnas.121187198
14. Bosseno M-F, Bernabé C, Gastélum EM, Kasten FL, Ramsey J, Espinoza B, et al. Predominance of *Trypanosoma cruzi* lineage I in Mexico. J Clin Microbiol. 2002;40:627–32. DOI: 10.1128/JCM.40.2.627-632.2002
15. Iwagami M, Higo H, Miura S, Yanagi T, Tada I, Kano S, et al. Molecular phylogeny of *Trypanosoma cruzi* from Central America (Guatemala) and a comparison with South American strains. Parasitol Res. 2007;102:129–34. DOI: 10.1007/s00436-007-0739-9

Address for correspondence: Dawn M. Roellig, Southeastern Cooperative Wildlife Disease Study, Department of Population Health, 589 DW Brooks Dr, Wildlife Health Bldg, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA; email: droellig@uga.edu

Proficiency of Nucleic Acid Tests for Avian Influenza Viruses, Australasia

Sacha Stelzer-Braid,*† Ros Escott,‡ Cristina Baleriola,* Peter Kirkland,§ Peter Robertson,* Michael Catton,¶ and William D. Rawlinson*†

An avian influenza quality assurance program was used to provide information for laboratories on the sensitivity and specificity of their avian influenza nucleic acid testing. Most laboratories were able to correctly detect clinically relevant amounts of influenza virus (H5N1), and results improved as each subsequent panel was tested.

Highly pathogenic avian influenza (HPAI) virus (H5N1) is endemic among the world's wild bird populations and continued to spread during 2006 to poultry across Asia, Africa, and mainland Europe (*1*,*2*). Sensitive, specific diagnostic methods are essential for early accurate detection of HPAI virus in the prepandemic and early pandemic phases in countries where no cases have been recorded, such as Australia (*3*).

Several sublineages of HPAI (H5N1) exist (*4*,*5*). Virus mutation requires that nucleic acid testing (NAT) methods such as reverse transcription–PCR (RT-PCR) be continually improved to remain sensitive for emerging strains (*6*–*12*). Currently, the World Health Organization (WHO) recommends an RT-PCR based on primers published in 1998 (www.who.int/csr/disease/avian_influenza/guidelines/RecAIlabtestsAug07.pdf).

The Study

We report results from an avian influenza quality assurance program (QAP) that used an established, Internet-based quality assurance reporting system (www.rcpaqap.com.au/serology), allowing remote data entry, rapid result dissemination, and expert comment. The QAP provided feedback to laboratories on NAT characteristics (PCR accuracy, sensitivity, and specificity), reporting optimization, and assessment of continuously updated laboratory-developed NAT methods.

During 2006, three panels of specimens were distributed to 29 participating laboratories: 15 from Australia (including 4 veterinary laboratories); 2 from Hong Kong Special Administrative Region, People's Republic of China; 5 from Singapore; 1 from New Caledonia; 1 from Malaysia; and 5 from New Zealand. The panels consisted of an Indonesian and a Vietnamese strain of avian influenza virus (H5N1), originally isolated from humans and grown in MDCK cells. Viral copy numbers were estimated by comparing real-time RT-PCR crossing-point values to a standard curve generated by using plasmid standards; the amplicon was cloned into pGEMT-Easy (Promega, Madison, WI, USA). Plasmid standard concentrations were estimated as described previously (*13*) and as recommended by the LightCycler manufacturer (Roche, Indianapolis, IN, USA). Sensitivity of NATs was determined with a range of clinically relevant nucleic acid concentrations of both influenza (H5N1) strains (10^3 to 10^{-1} copies/μL) to enable laboratories to assess limit of detection (LOD) of their assays. Specificity was assessed by inclusion of other influenza strains and a negative control (Table 1). All strains and MDCK cells were inactivated by exposure to 50 KGy of γ-irradiation, except for strain A (H7N4), which was inactivated by the addition of lysis buffer (*14*).

Four experiments to define optimum conditions were conducted. 1) LOD determinations, with a dilution series of all strains, were tested by using real-time RT-PCR (*15*). 2) Transport media were compared by using serial dilutions of inactivated influenza virus (H5N1) in phosphate-buffered saline with gelatin (with antimicrobial agents) (PBSG), TE buffer, and buffer RLT (lysis) (QIAGEN, Valencia, CA, USA), placed at –80°C, –20°C, +4°C, +25°C, and +37°C for 10 days. Each day, 1 tube at each temperature condition was removed, and viral DNA was extracted by using the QIAamp viral RNA minikit (QIAGEN) and tested with a real-time RT-PCR (*15*). 3) For further stability testing, a test panel diluted in PBSG was sent by courier from Sydney to Hong Kong, held by Australian customs for 7 days, and returned unopened 15 days after dispatch. The temperature range the shipped panel was exposed to is unknown; however, previous temperature loggers have recorded temperatures from 22°C to 33°C. The panel that traveled was tested against a panel that had been stored optimally (–80°C) (*15*). No difference was detected in the amount of virus in the specimens that traveled compared with optimally stored specimens, indicating that the specimens were stable under normal transport conditions (results not shown). 4) Homogeneity was established before distribution by having the

*Prince of Wales Hospital, Randwick, New South Wales, Australia; †University of New South Wales, Kensington, New South Wales, Australia; ‡Royal College of Pathologists of Australasia, North Ryde, New South Wales, Australia; §Elizabeth Macarthur Agricultural Institute, Camden, New South Wales, Australia; and ¶Victorian Infectious Diseases Reference Laboratory, North Melbourne, Victoria, Australia

DOI: 10.3201/eid1407.071098

Table 1. Avian influenza quality assurance panel specimen details, Australasia

Specimen	Dilution	Copy number/μL
Influenza A (H5N1) Indonesian	1:1,000	6.25×10^3
Influenza A (H5N1) Vietnamese	1:1,000	5×10^3
Influenza A (H5N1) Indonesian	1:100,000	6.25×10^1
MDCK-negative control	1:1,000	NA
Influenza A (H5N1) Vietnamese	1:100,000	5×10^1
Influenza A (H3N2) (isolated in Sydney, New South Wales, Australia; close sequence match to A/Canterbury/29/2005)	1:1,000	1.25×10^2
Influenza B (isolated in Sydney; no sequence information available)	1:1,000	2×10^2
Influenza A (H7N4) (A/emu/NSW/97)	1:1,000	1×10^4
Influenza A (H5N1) Indonesian*	1:10,000,000	6.25×10^{-1}
Influenza A (H5N1) Vietnamese*	1:10,000,000†	5×10^{-1}

*These dilutions were included in panels 2 and 3 only; NA, not applicable.
†For panel 3, the dilution for the influenza (H5N1) Vietnamese strain was changed to 1:1,000,000, with a copy number of 5×10^0 μL.

panel tested and approved by 2 reference laboratories (Victorian Infectious Diseases Laboratory, Melbourne, Victoria, Australia; Western Australian Centre for Pathology and Medical Research, Perth, Western Australia, Australia) and 1 animal reference laboratory (Elizabeth Macarthur Agricultural Institute, Camden, New South Wales, Australia).

For each panel the samples were diluted in PBSG and transported by courier at ambient temperature in 3 seasons (autumn, winter, summer). Participants were not required to use a certain NAT method. Participants were asked for information on methods used, including extraction and RT-PCR protocol and primer/probe sequences. A total of 780 results were analyzed, and a report was issued to participants within 3 weeks of each survey closing, well before the next panel shipment. This allowed participants to adjust their testing procedures if necessary before the next survey began. Results were reported by participants as positive, negative, or equivocal. For simplicity, we report equivocal results as positive, given that participating laboratories retest an equivocal result and generally do not report such results as negative. On average for the 3 panels, 2.6% of results were reported as equivocal.

Panel 1 contained 8 specimens, which included 2 dilutions (10^3 and 10^1 copies/μL) of each subtype H5N1 strain (Table 1). Only 35% of participants correctly identified all samples; 95% reported a correct result for the highest concentration of both subtype H5N1 strains (10^3 copies/μL); 70% could detect the lower concentration (10^1 copies/μL). Only 46% of participating laboratories used an influenza A matrix assay as well as a specific H5 assay (Table 2). Laboratories were advised to use both methods in tandem to reduce the chance of missing variant influenza (H5N1) strains that might not be detected by their specific H5 assay. Some false positives (1.3%) were reported, and some confusion regarding terminology occurred: many laboratories reported results as for subtype H5N1 assays, despite most of these results being specific for the H5 gene only.

For panel 2, no participants correctly identified all samples because of the addition of 2 extremely dilute samples of influenza virus (H5N1) (10^0 and 10^{-1} copies/μL) that were below the LOD for most laboratories. Eleven percent of participants detected 1 strain of HPAI virus (H5N1) by using primers specific for H5 or subtype H5N1 at 1 of the 2 highest dilutions, but not both. In our experience, dilute specimens are useful for assessing the LOD of the testing system because they may highlight the most sensitive methods available. The number of laboratories using a generic influenza A test, in addition to a specific H5 test, increased to 73% (Table 2).

For panel 3, sensitivity of detection improved compared with panel 2: 25% of participants detected a strain of influenza virus (H5N1) at the lowest concentrations. Sensitivity of H5/H5N1 testing for the influenza (H5N1) Vietnamese strain increased over time, while sensitivity of

Table 2. Summary of avian influenza quality assurance project, Australasia*

Panel no.†	Date	% Correct results, Indonesian strain‡	% Correct results, Vietnamese strain‡	% Testing for influenza A matrix	Most common extraction method (% participants)	Most common amplification method (% participants)
1	2006 Oct 7	87.5	80	46	QIAGEN QIAamp viral RNA minikit (50)	QIAGEN Artus Influenza/H5 LC RT-PCR kit (20)
2	2006 Nov 9	84	84	73	QIAGEN QIAamp viral RNA minikit (50)	Invitrogen Superscript III qRT-PCR (31)
3	2006 Jun 11	82	88	75	QIAGEN QIAamp viral RNA minikit (50)	Invitrogen Superscript III qRT-PCR (40)

*RT-PCR, reverse transcription–PCR.
†Panels 2 and 3 had 2 very low dilutions of subtype H5N1 that were beyond the limit of detection for most laboratories. Percentage of correct results reported for the Indonesian and Vietnamese strains, with these results included, is 59 and 59 for panel 2 and 56 and 66 for panel 3, respectively.
‡Results reported for influenza H5/(H5N1) testing.

testing decreased slightly over the 3 panels for the influenza (H5N1) Indonesian strain (Table 2). Laboratories had altered primer/probe sets to increase sensitivity for the Vietnamese strain, which resulted in decreased sensitivity for the Indonesian strain. Sensitivities of other testing methods (influenza A, B, H3) increased during subsequent testing of each panel (data not shown); the number of correct results reported by participants using influenza A matrix testing rose from 84% in panel 1 to 91% in panel 3.

Conclusions

Most participants did not disclose their primer/probe sequence information, which made it difficult to recommend the most sensitive methods to other participants. However, during a prepandemic phase, having a range of primers/probes being used may be optimal, providing influenza A matrix detection is also conducted and QA is maintained, until WHO recommends a method to detect new pandemic strains.

Participants in the avian influenza QAP made clear improvements in the sensitivity and specificity of their NAT methods over time. It is important to provide continuing QA to expose inconsistencies in results or primers that may be skewed toward a particular strain.

Acknowledgments

We thank all the laboratories participating in the Influenza QAP, the RCPA Quality Assurance Programs Pty Ltd (Serology); National Health and Medical Research Council for support, and Michael Fennell, Paul Selleck, and Julian Druce for assistance with influenza strains.

Dr Stelzer-Braid is a postdoctoral scientist in virology research at the Prince of Wales Hospital in Sydney. Her research interests include emerging viruses, hepatitis C virus, and new methods for detecting respiratory viruses.

References

1. Human deaths from avian influenza in Azerbaijan—further spread in birds worldwide. Euro Surveillance. European Communicable Disease Bulletin. 2006;11:E060316.1.
2. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA, et al. Global patterns of influenza A virus in wild birds. Science. 2006;312:384–8. DOI: 10.1126/science.1122438
3. Hurt AC, Hansbro PM, Selleck P, Olsen B, Minton C, Hampson AW, et al. Isolation of avian influenza viruses from two different transhemispheric migratory shorebird species in Australia. Arch Virol. 2006;151:2301–9. DOI: 10.1007/s00705-006-0784-1
4. Smith GJ, Naipospos TS, Nguyen TD, de Jong MD, Vijaykrishna D, Usman TB, et al. Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. Virology. 2006;350:258–68. DOI: 10.1016/j.virol.2006.03.048
5. Ducatez MF, Olinger CM, Owoade AA, Tarnagda Z, Tahita MC, Sow A, et al. Molecular and antigenic evolution and geographical spread of H5N1 highly pathogenic avian influenza viruses in western. J Gen Virol. 2007;88:2297–306. DOI: 10.1099/vir.0.82939-0
6. Hoffmann B, Harder T, Starick E, Depner K, Werner O, Beer M, et al. Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR. J Clin Microbiol. 2007;45:600–3. DOI: 10.1128/JCM.01681-06
7. Ng LF, Barr I, Nguyen T, Noor SM, Tan RS, Agathe LV, et al. Specific detection of H5N1 avian influenza A virus in field specimens by a one-step RT-PCR assay. BMC Infect Dis. 2006;6:40. DOI: 10.1186/1471-2334-6-40
8. Wei HL, Bai GR, Mweene AS, Zhou YC, Cong YL, Pu J, et al. Rapid detection of avian influenza virus a and subtype H5N1 by single step multiplex reverse transcription-polymerase chain reaction. Virus Genes. 2006;32:261–7. DOI: 10.1007/s11262-005-6910-4
9. Xie Z, Pang YS, Liu J, Deng X, Tang X, Sun J, et al. A multiplex RT-PCR for detection of type A influenza virus and differentiation of avian H5, H7, and H9 hemagglutinin subtypes. Mol Cell Probes. 2006;20:245–9. DOI: 10.1016/j.mcp.2006.01.003
10. 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. DOI: 10.1016/j.jviromet.2005.08.004
11. Whiley DM, Sloots TP. A 5′-nuclease real-time reverse transcriptase-polymerase chain reaction assay for the detection of a broad range of influenza A subtypes, including H5N1. Diagn Microbiol Infect Dis. 2005;53:335–7. DOI: 10.1016/j.diagmicrobio.2005.08.002
12. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 2002;40:3256–60. DOI: 10.1128/JCM.40.9.3256-3260.2002
13. Sato S, Noguchi Y, Wada H, Fujita S, Nakamura S, Tanaka R, et al. Quantitative real-time RT-PCR analysis of NY-ESO-1 and LAGE-1a mRNA expression in normal tissues and tumors, and correlation of the protein expression with the mRNA copy number. Int J Oncol. 2005;26:57–63.
14. Blow JA, Dohm DJ, Negley DL, Mores CN. Virus inactivation by nucleic acid extraction reagents. J Virol Methods. 2004;119:195–8. DOI: 10.1016/j.jviromet.2004.03.015
15. Heine HG, Trinidad L, Selleck PW, Lowther S. Rapid detection of highly pathogenic avian influenza H5N1 by TaqMan RT-PCR. Avian Dis. 2007;51:370–2. DOI: 10.1637/7587-040206R.1

Address for correspondence: William D. Rawlinson, Virology Division, Department of Microbiology, South Eastern Area Laboratory Services, Prince of Wales Hospital, Randwick, NSW 2031, Australia; email: w.rawlinson@unsw.edu.au

New *qnr* Gene Cassettes Associated with Superintegron Repeats in *Vibrio cholerae* O1

Érica L. Fonseca,* Fernanda dos Santos Freitas,* Verônica V. Vieira,† and Ana C.P. Vicente*

A novel *qnr* determinant emerged in ciprofloxacin-resistant *Vibrio cholerae* O1 from the Amazon region of Brazil. This *qnr*VC1 was in a typical class 1 integron. Its *attC* showed 89% identity with *V. parahaemolyticus* superintegron repeats. Analysis showed *V. cholerae* O1 carrying *qnr*VC2 associated with a *V. cholerae* superintegron repeat.

Quinolones are antimicrobial drugs effective against gram-negative bacteria. These drugs have been widely used as alternatives to conventional drug therapy for treating *Mycobacterium* spp. and *Neisseria* spp. infections. Quinolone resistance is frequently caused by mutations in DNA gyrase and topoisomerase IV chromosomal genes and an increased activity of efflux pumps that reduce intracellular concentrations of the drug (*1*).

Resistance to these drugs has been attributed to plasmid-mediated *qnr* genes. These genes are found mainly in *Enterobacteriaceae* and affect the dynamics of development and acquisition of quinolone resistance. Until now, all plasmid-borne *qnr* determinants were associated with atypical *sul*-type integrons, which are characterized by a duplication of a 3′ conserved segment, and a putative recombinase known as open reading frame (ORF) 513 (*2*). In contrast to typical gene cassettes, these *qnr* genes lack the *attC* site and, consequently, are unable to move by class 1 integrase excision. Qnr-like proteins coded by chromosomal genes have been found in *Vibrionaceae*, and this family has been identified as the source of *qnr* genes (*3*,*4*). However, no *qnr* homolog was found in any *Vibrio cholerae* genome sequenced.

Members of the family *Vibrionaceae* are characterized by superintegrons (SIs), which are chromosomal genetic elements containing a variety of gene cassettes with nearly identical *attC* sites (e.g., *V. cholerae* SI repeats) (*5*). We report 2 new *qnr*-like genes (*qnr*VC1 and *qnr*VC2) in typical class 1 integrons from clinical strains of *V. cholerae* O1.

The Study

V. cholerae O1 isolates from the cholera epidemic in Brazil (1991–2000) were screened for antimicrobial drug resistance and class 1 integrons. VC627, a clinical strain from the Amazon region isolated in 1998, was resistant to ciprofloxacin, in contrast to other isolates. This strain was the only one positive for class 1 integrase but not for *qac*EΔ1 and *sul*I genes (*6*). Sequence analysis of the variable region of this class 1 integron showed an *aad*A2 gene cassette and an ORF similar to *qnr* determinants. Its deduced amino acid sequence showed 69% sequence identity with a chromosomal protein of *Photobacterium profundum*, which was recently identified as a quinolone-resistance determinant (QnrPP) (*3*) and 57% identity with QnrA1, a plasmid-encoded protein found in *Enterobacteriaceae* (*2*). This new *qnr*-like gene in *V. cholerae* was named *qnr*VC1 (EU436855).

Qnr proteins belong to the pentapeptide repeat family, which consists of uninterrupted pentapeptide repeats comprising 92% of the sequence and play a role in DNA gyrase protection (*7*). These repeat features were observed in the QnrVC1 deduced amino acid sequence with the consensus sequence [AC][DN][LF]XX (amino acids in brackets are found more frequently and XX indicates other amino acids) (*8*) (online Appendix Figure, available from www.cdc.gov/EID/content/14/7/1129-appG.htm), with a large proportion of serine residues in the fourth position (first X), as observed by Robicsek et al. (*2*).

The *qnr*VC homologs in chromosomes of other bacterial species were investigated by using BLAST analysis (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Similar to other reports of such homologs (*3*,*4*), we found Qnr-like proteins in recently published genomes from different families, including other members of *Vibrionaceae* (Figure). Analysis in conjunction with informatics capabilities (in silico analysis) showed a 657-nt sequence in a class 1 integron of *V. cholerae* O1 isolated in Vietnam in 2004 (AB200915). This ORF had 75% nt similarity with *qnr*VC1, and it was designated *qnr*VC2.

The *qnr*VC gene cassettes are characterized by *attC* sites, which are absent in all others *qnr* genes already identified. Supporting the hypothesis of *Vibrionaceae* as the source of *qnr* genes (*3*), the *qnr*VC1 *attC* site has 89% identity with *V. parahaemolyticus* repeats, and the *qnr*VC2 putative *attC* site has 96% identity with a *V. cholerae* repeat sequence, both of which are characteristic structures of SIs. Rowe-Magnus et al. (*5*) reported that SI repeat recombination sites are species specific and these elements would be the source of gene cassettes found in drug-resistance integrons, such as class 1 integrons. Therefore, we hypothesize

*Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; and †Instituto Nacional de Controle de Qualidade em Saúde, Rio de Janeiro

DOI: 10.3201/eid1407.080132

that *qnr*VC1 gene cassette could have originated from a *V. parahaemolyticus* SI, whereas *qnr*VC2 originated in *V. cholerae*. Curiously, no chromosomal *qnr* genes identified in *Vibrionaceae* were associated with SIs.

The *qnr*VC1 gene showed high amino acid divergence compared with all *qnr* genes described, with similarities ranging from 44% to 69%. The *qnr*VC1 had a G + C content of 36.8%, which is considerably different from that of the *V. cholerae* genome (47.5%) and is evidence of horizontal gene transfer.

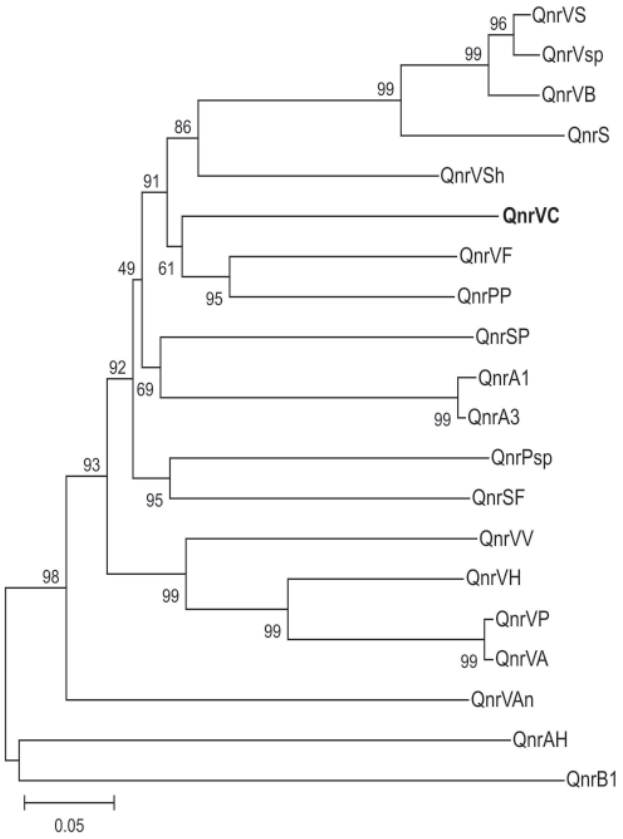


Figure. Genetic relationships of plasmid- and chromosome-encoded *qnr* proteins. Species and GenBank accession nos. are as follows. QnrVS (*Vibrio splendidus*, EAP95542), QnrVsp (*Vibrio* sp., EAQ55748), QnrS1 (*Shigella flexneri*, BAD88776), QnrVC (*V. cholerae*, strain 627; EU436855; this work, shown in **boldface**); QnrPP (*Photobacterium profundum*, YP132629), QnrVF (*V. fisheri*, AAW85819), QnrSP (*Shewanella pealeana*, EAV99957), QnrA1 (*Escherichia coli*, AAY46800), QnrA3 (*S. algae*, AAZ04782), QnrPsp (*Psychromonas* sp., EAS39797), QnrSF (*S. frigidimarina*, ABI71948), QnrVV (*V. vulnificus*, AAO07889), QnrVP (*V. parahaemolyticus*, BAC61438), QnrVA (*V. alginolyticus*, EAS75285), QnrVAn (*V. angustum*, EAS64891), QnrAH (*Aeromonas hydrophila*, ABK38882), QnrB1 (*Klebsiella pneumoniae*, ABG82188), QnrVSh (*V. shilonii*, EDL55273), QnrVB (*Vibrionales bacterium*, EDK31146), QnrVH (*V. harveyi*, EDL69958). Support of the branching order was determined by 1,000 interior branch test replicates. The distance-based tree was generated by using p distance with the neighbor-joining method with MEGA version 3.1 (www.megasoftware.net). Values along the horizontal lines are the interior-branch test percentages after testing 1,000 topologies. Scale bar indicates the number of substitutions per alignment site, which is reflected by branch lengths.

Promoter Pc, which is found in the 5′ conserved segment of the integron carrying *qnr*VC1, was identical to the hybrid configuration described (*9*,*10*) and is defined by 1 point mutation in the canonical –35 region. The hybrid Pc version has 20-fold lower promoter activity than that of the wild-type (strong) promoter. However, another study verified expression of genes inserted into integrons under the control of a hybrid Pc configuration (*11*). Moreover, in silico analysis detected a putative promoter (–35 TTGAGA [17 nt] –10 TAGTCT) in the 5′ untranslated region of the *qnr*VC1 gene cassette. Therefore, our findings characterize conditions for *qnr*VC1 expression in the class 1 integron.

Quinolones have excellent antimicrobial activity against *V. cholerae* (*12*). Strain VC627 shows a 10-fold increase in resistance to ciprofloxacin (MIC 0.25 μg/mL) compared with strains from the seventh pandemic lineage in Brazil (MIC 0.02 μg/mL). This increased resistance was also observed in a *Shigella flexneri* strain harboring *qnr*S (*13*,*14*) and *V. splendidus* harboring chromosomal *qnr*VS1 and *qnr*VS2 (*4*). This MIC value resembles that caused by *gyr*A mutations in *V. cholerae* ciprofloxacin-resistant strains (*15*). Therefore, dissemination of *qnr* genes by lateral gene transfer may determine, in a 1-step fashion, the same drug-resistance profile caused by acquired mutations in housekeeping genes.

Conclusions

Our study reports characterization of 2 new *qnr* genes associated with SI *attC* sites in typical class 1 integrons and their emergence in *V. cholerae*. These genetic features have not been observed in either chromosomal- or plasmid-borne *qnr* determinants already described.

Our study suggests that, at least in *Vibrionaceae*, quinolone-resistance genes were mobilized from SIs by class 1 integrase activity because *qnr*VC1 and *qnr*VC2 *attC* elements are similar to *V. parahaemolyticus* and *V. cholerae* SI repeats. These findings corroborate the hypothesis that SIs are a source of cassettes present in drug-resistance integrons (*5*). These elements are part of a reasonable scenario for a 1-step acquisition of quinolone resistance by bacteria (e.g., *Mycobacterium* spp. and *Neisseria* spp.). This acquisition may jeopardize treatment and control and have adverse consequences on infections caused by these organisms.

This work was supported by a fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico, a grant from the Instituto Oswaldo Cruz, and the Plataforma de Desenvolvimento Technólogico em Insumos para a Saúde for sequencing.

Ms Fonseca is a doctoral student at the Laboratório de Genética Molecular de Microrganismos, Instituto Oswaldo Cruz, FI-

OCRUZ, in Rio de Janeiro. Her research interest is genetic variability of microorganisms, especially bacteria of clinical relevance.

References

1. Hawkey PM. Mechanisms of quinolone action and microbial response. J Antimicrob Chemother. 2003;51:29–35. DOI: 10.1093/jac/dkg207
2. Robicsek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect Dis. 2006;6:629–40. DOI: 10.1016/S1473-3099(06)70599-0
3. Poirel L, Liard A, Rodriguez-Martinez J-M, Nordmann P. *Vibrionaceae* as a possible source of Qnr-like quinolone resistance determinants. J Antimicrob Chemother. 2005;56:1118–21. DOI: 10.1093/jac/dki371
4. Cattoir V, Poirel L, Mazel D, Soussy C-J, Nordmann P. *Vibrio splendidus* as the source of plasmid-mediated QnrS-like quinolone resistance determinants. Antimicrob Agents Chemother. 2007;51:2650–1. DOI: 10.1128/AAC.00070-07
5. Rowe-Magnus DA, Guérout A-M, Ploncard P, Dychinco B, Davies J, Mazel D. The evolutionary history of chromosomal super-integrons provides an ancestry for multiresistant integrons. Proc Natl Acad Sci U S A. 2001;98:652–7. DOI: 10.1073/pnas.98.2.652
6. Fonseca EL, Vieira VV, Cipriano R, Vicente AC. Class 1 integrons in *Pseudomonas aeruginosa* isolates from clinical settings in Amazon region, Brazil. FEMS Immunol Med Microbiol. 2005;44:303–9. DOI: 10.1016/j.femsim.2005.01.004
7. Vetting MW, Hegde SS, Fajardo JE, Fiser A, Roderick SL, Takiff HE, et al. Pentapeptide repeat proteins. Biochemistry. 2006;45:1–10. DOI: 10.1021/bi052130w
8. Tran JH, Jacoby GA. Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci U S A. 2002;99:5638–42. DOI: 10.1073/pnas.082092899
9. Collis CM, Hall RM. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob Agents Chemother. 1995;39:155–62.
10. Lévesque C, Brassard S, Lapointe J, Roy PH. Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. Gene. 1994;142:49–54. DOI: 10.1016/0378-1119-(94)90353-0
11. Dubois V, Poirel L, Marie C, Arpin C, Nordmann P, Quentin C. Molecular characterization of a novel class 1 integron containing bla(GES-1) and a fused product of *aac*3-Ib/*aac*6′-Ib′ gene cassettes in *Pseudomonas aeruginosa.* Antimicrob Agents Chemother. 2002;46:638–45. DOI: 10.1128/AAC.46.3.638-645.2002
12. Yamamoto T, Nair GB, Albert MJ, Parodi CC, Takeda Y. Survey of in vitro susceptibilities of *Vibrio cholerae* O1 and O139 to antimicrobial agents. Antimicrob Agents Chemother. 1995;39:241–4.
13. Hata M, Suzuki M, Matsumoto M, Takahashi M, Sato K, Ibe S, et al. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob Agents Chemother. 2005;49:801–3. DOI: 10.1128/AAC.49.2.801-803.2005
14. Martínez-Martínez L, Pascual A, García I, Tran J, Jacoby GA. Interaction of plasmid and host quinolone resistance. J Antimicrob Chemother. 2003;51:1037–9. DOI: 10.1093/jac/dkg157
15. Baranwal S, Dey K, Ramamurthy T, Nair GB, Kundu M. Role of active efflux in association with target gene mutations in fluoroquinolone resistance in clinical isolates of *Vibrio cholerae*. Antimicrob Agents Chemother. 2002;46:2676–8. DOI: 10.1128/AAC.46.8.2676-2678.2002

Address for correspondence: Érica L. Fonseca, Laboratório de Genética Molecular de Microrganismos, Pavilhão Leônidas Deane, Sala 607, Instituto Oswaldo Cruz, FIOCRUZ, Avenida Brazil 4365, PO Box 926, Manguinhos, Rio de Janeiro, Brazil; email: ericafon@ioc.fiocruz.br

Experimental Infection of Cattle with Highly Pathogenic Avian Influenza Virus (H5N1)

Donata Kalthoff,* Bernd Hoffmann,* Timm Harder,* Markus Durban,* and Martin Beer*

Four calves were experimentally inoculated with highly pathogenic avian influenza virus A/cat/Germany/R606/2006 (H5N1) isolated from a cat in 2006. All calves remained healthy, but several animals shed low amounts of virus, detected by inoculation of nasal swab fluid into embryonated chicken eggs and onto MDCK cells. All calves seroconverted.

Since 1997, an epidemic of highly pathogenic avian influenza virus (HPAIV) subtype H5N1 has spread in Asia, causing fatal infections in poultry, wild birds, and mammals, including humans (*1*). Knowing the susceptibility to HPAIV (H5N1) of mammalian species living in close proximity to humans and poultry, such as members of the family *Bovidae* (e.g., cattle or water buffalo), would be helpful to those developing surveillance plans or determining risk areas. Serologic examinations have indicated that calves might be susceptible to influenza A virus (*2*); however, so far only 1 strain (A/calf/Duschanbe/55/71 H3N2) has been identified as a cattle strain (*3*). A correlation between influenza A virus infection, reduced milk yield, and respiratory symptoms in dairy cows was assumed in the late 1990s (*4*,*5*) and has received recent attention (*6*,*7*). Nevertheless, to our knowledge, no data about the susceptibility of cattle to infections with HPAIV have been reported. Therefore, we experimentally infected bovine calves with HPAIV (H5N1) and collected data about clinical symptoms, viral excretion, and serologic reactions.

The Study

In 2007, 6 Holstein-Friesian calves, 3 month of age, were obtained from a breeder near Greifswald-Insel Riems, Germany. Their influenza A virus–free status was confirmed, and no avian influenza virus–specific genomes or antibodies were detected. All experiments were performed in the high-containment animal facility (Biosafety Level 3+) at the Friedrich-Loeffler-Institut (trial approval no. LVLM-V/TSD/7221.3-1.1-003/07).

Four of the calves were intranasally inoculated with HPAIV (H5N1) strain A/cat/Germany/R606/2006, which had been isolated from a cat in 2006 (*8*,*9*). The virus was aerosolized in 5 mL cell culture medium containing $10^{8.5}$ 50% egg infectious dose/mL (third egg passage). The other 2 calves (contacts) were not inoculated but were housed in the same containment room for the duration of the experiment. For 7 days all calves were monitored by physical examination, and pharyngeal swabs were collected and examined for virus excretion.

All 6 calves remained healthy; no specific clinical signs were observed. All nasal swabs were tested with real-time reverse transcription–PCR specific for subtype H5N1 (*10*), and the genomic load was semiquantified by using threshold cycle values. Infectious virus was detected by inoculation of swab fluid into embryonated chicken eggs with 1 subsequent passage and onto MDCK cells (collection of cell lines in veterinary medicine, Friedrich-Loeffler-Institut, Isle of Riems, RIE83).

Nasal swabs from all inoculated calves collected at 1 day postinoculation (dpi) were positive for viral RNA, and 3 of 4 calves shed infectious virus, detected by virus isolation in embryonated chicken eggs (Table 1). Isolation of infectious virus in cell culture was successful in 2 of 4 samples. Furthermore, of 4 inoculated calves, 2 were positive for HPAIV (H5N1) genome copies at 2 dpi, and 1 shed infectious virus. From 3 dpi through 7 dpi, samples from nasal swabs of all animals were negative for viral genome, and all nasal swabs of the 2 contact calves remained negative for HPAIV (H5N1) RNA during the experiment (Table 1).

Assuming that susceptible animals should mount an antibody response, we looked for antibodies against the highly conserved and immunogenic nucleoprotein (NP) of type A influenza viruses (*11*). Heat-inactivated (30 min at 56°C) serum samples collected at 0, 7, 14, 21, 28, and 91 dpi were tested for NP-specific antibodies with a licensed commercial ELISA (avian influenza A–blocking ELISA, Pourquier, Montpellier, France) according to the manufacturer's instructions. Serum samples from 50 HPAIV (H5N1)–negative, nonrelated cattle were used as controls and to confirm the specificity of the ELISA (data not shown).

To quantify the serologic response, we performed virus neutralization (VN) and hemagglutination inhibition (HI) tests with homotypic virus. The VN test was modified according to a previously described procedure (*12*). In brief, bovine serum samples were heat inactivated for 30 min at 56°C, and 3-fold serial dilutions were performed in a 50-μL volume of cell culture medium in 96-well plates. The diluted serum samples were mixed with an equal volume of media containing homotypic influenza virus at a concentration

*Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

DOI: 10.3201/eid1407.071468

Table 1. Detection of highly pathogenic avian influenza virus (H5N1) in nasal swabs from calves, Germany, 2007*

	Day postinoculation							
	0	1			2			3–7
Calf	PCR†	PCR	Egg	Cell	PCR	Egg	Cell	PCR
Inoculated								
A1	>40	20–30	–	–	30–40	–	–	>40
A2	>40	30–40	+	+	30–40	+	–	>40
A3	>40	20–30	+	+	>40	ND	ND	>40
A4	>40	30–40	+	–	>40	ND	ND	>40
Contact								
K1	>40	>40	ND	ND	>40	ND	ND	>40
K2	>40	>40	ND	ND	>40	ND	ND	>40

*Inoculated calves received highly pathogenic influenza virus (H5N1) strain A/cat/Germany/R606/2006 (*8*,*9*); contact calves were not inoculated but were housed with the inoculated calves. Egg, embryonated chicken eggs; cell, MDCK cell culture; +, virus detected; –, virus not detected; ND, not done.
†H5-specific real-time reverse transcription–PCR; results are shown as range of detected threshold cycle values.

of 10^2 50% tissue culture infectious dose/well. After 1 h incubation at 37°C in a 5% CO_2 humidified atmosphere, 100 µL of Vero cells (African green monkey kidney, collection of cell lines in veterinary medicine, Friedrich-Loeffler-Institut, Isle of Riems, RIE228) at 1.5×10^5/mL was added to each well. The plates were incubated for 3 days at 37°C and 5% CO_2. Viral replication was assessed by visually scoring the cytopathic effect without staining. Each assay was validated by comparison with positive and negative control serum from chickens and cattle as well as back titration of the used virus dilutions. Previous treatment with periodate (*13*) had no influence on the neutralizing titers. Serum samples were also tested for H5-specific antibodies by HI test with 4 hemagglutinating units of homotypic virus as antigen according to standardized methods (*14*). All serum samples were treated with periodate and heat inactivated to eliminate serum inhibitors. The HI tests were performed with a starting dilution of 1:8 by using a 1% suspension of chicken erythrocytes in a 0.85% saline solution.

The commercial NP-specific ELISA detected influenza A virus–specific antibodies at 14 dpi in 2 of the 4 inoculated animals (Table 2). All 4 animals had positive scores for neutralizing antibodies against the homologous virus at 14 dpi; specific titers ranged from 16 to 51. Furthermore, the HI test detected titers of 8 at 28 dpi in the 4 inoculated calves. At 21 dpi, VN testing indicated that 1 of the contact calves was positive for subtype H5N1–specific antibodies, NP-ELISA results for this calf were questionable, and HI testing did not detect any hemagglutinating antibodies. The other contact calf remained serologically negative throughout the experiment. Finally, 3 months after inoculation, VN test and ELISA clearly indicated seroconversion by all inoculated calves and 1 of the contact calves. In contrast, HI results were negative for all but 2 inoculated animals (Table 2).

Conclusions

Our findings show that HPAIV (H5N1) has the potential to infect bovine calves, at least after high-titer intranasal inoculation, and that conventional HI tests may underestimate such infections. Furthermore, asymptomatic shedding of HPAIV (H5N1) by infected calves and subsequent seroconversion seem to be possible, and even low levels of HPAIV (H5N1) might be sufficient to induce a detectable antibody response in contact calves. However, the possibility that the infectivity detected in the contact

Table 2. Serologic testing results for highly pathogenic avian influenza virus (H5N1) in calves, Germany, 2007*

	Day postinoculation																	
	0			7			14			21			28			91		
Calf	NP†	VN‡	HI§	NP	VN	HI	NP	VN	HI	NP	VN	HI	NP	VN	HI	NP	VN	HI
Inoculated																		
A1	98	2.3	<3	90	3.3	<3	42	**4**	<3	**26**	**4**	<3	**24**	**6.2**	**3**	**20**	**7**	<3
A2	114	0.4	<3	117	3	<3	69	**5**	<3	**27**	**4**	<3	**22**	**6.2**	**3**	**18**	**6.7**	**3**
A3	118	<1	<3	90	2.7	<3	**28**	**5.7**	<3	**29**	**5**	<3	**21**	**7**	**3**	**15**	**7**	**3**
A4	102	0.7	<3	122	2	<3	**25**	**5.3**	<3	**24**	**5**	<3	**24**	**5.8**	**3**	**19**	**6.3**	<3
Contact																		
K1	96	1.3	<3	88	<1	<3	73	2	<3	44	**4**	<3	40	**4.5**	<3	45	**5**	<3
K2	120	0.7	<3	98	2.3	<3	89	<1	<3	68	<1	<3	76	0.7	<3	50	<1	<3

*Inoculated calves received highly pathogenic influenza virus (H5N1) strain A/cat/Germany/R606/2006 (*8*,*9*); contact calves were not inoculated but were housed with the inoculated calves. Positive results are in **boldface**.
†NP, avian influenza A– blocking ELISA against nucleoprotein (Pourquier, Montpellier, France) inhibition % (<35, positive; >45, negative; 35–45, questionable).
‡VN, virus neutralization test (ND100 $\log_2$); values ≥4 are considered positive.
§HI, hemagglutination inhibition ($\log_2$); values ≥3 are considered positive.

calf at 1 dpi was the result of residual inoculum cannot be ruled out. Although the question whether calf-to-calf transmission of HPAIV (H5N1) occurs could not be definitely answered by our study, bird-to-calf transmission resulting in seroconversion is probable.

The incidence of clinical infections of cattle with HPAIV (H5N1) in disease-endemic regions should be low. However, our data indicate that serum from bovine species would be a valuable source of additional information about transmission events, especially in regions like Asia and Egypt, where HPAIV (H5N1) is endemic and probability of contact between poultry and cattle is high. The NP-ELISA is currently the assay of choice for the evaluation of bovine serum, and the VN test should be used for confirmation.

Acknowledgments

We thank M. Grawe for excellent technical assistance, and T. Arnold and G. Bauer for caring for the cattle during the challenge study.

This study was funded by the Federal Ministry of Food, Agriculture and Consumer Protection, Germany (FSI project no. 1-4.1).

Dr Kalthoff is a veterinarian at the Institute of Diagnostic Virology, FLI Insel Riems. Her research interests are the pathogenesis of HPAIV in different bird and mammalian species and vaccine development.

References

1. Webster RG, Peiris M, Chen H, Guan Y. H5N1 outbreaks and enzootic influenza. Emerg Infect Dis. 2006;12:3–8.
2. Lopez JW, Woods GT. Influenza virus in ruminants: a review. Res Commun Chem Pathol Pharmacol. 1984;45:445–62.
3. Campbell CH, Easterday BC, Webster RG. Strains of Hong Kong influenza virus in calves. J Infect Dis. 1977;135:678–80.
4. Brown IH, Crawshaw TR, Harris PA, Alexander DJ. Detection of antibodies to influenza A virus in cattle in association with respiratory disease and reduced milk yield. Vet Rec. 1998;143:637–8.
5. Gunning RF, Brown IH, Crawshaw TR. Evidence of influenza A virus infection in dairy cows with sporadic milk drop syndrome. Vet Rec. 1999;145:556–7.
6. Graham DA, Calvert V, McLaren E. Retrospective analysis of serum and nasal mucus from cattle in Northern Ireland for evidence of infection with influenza A virus. Vet Rec. 2002;150:201–4.
7. Crawshaw TR, Brown IH, Essen SC, Young SC. Significant rising antibody titres to influenza A are associated with an acute reduction in milk yield in cattle. Vet J. 2007 Sep 7 [Epub ahead of print]. DOI: 10.1016/j.tvjl.2007.07.022
8. Weber S, Harder T, Starick E, Beer M, Werner O, Hoffmann B, et al. Molecular analysis of highly pathogenic avian influenza virus of subtype H5N1 isolated from wild birds and mammals in northern Germany. J Gen Virol. 2007;88:554–8. DOI: 10.1099/vir.0.82300-0
9. Giese M, Harder TC, Teifke JP, Klopfleisch R, Breithaupt A, Mettenleiter TC, et al. Experimental infection and natural contact exposure of dogs with avian influenza virus (H5N1). Emerg Infect Dis [serial on the Internet]. 2008 Feb [cited 2008 Apr 15]. Available from http://www.cdc.gov/EID/content/14/2/308.htm
10. Hoffmann B, Harder T, Starick E, Depner K, Werner O, Beer M. Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR. J Clin Microbiol. 2007;45:600–3. DOI: 10.1128/JCM.01681-06
11. Paltrinieri S, Spagnolo V, Giordano A, Moreno Martin A, Luppi A. Influenza virus type A serosurvey in cats [letter]. Emerg Infect Dis [serial on the Internet]. 2007 Apr [cited 2008 Apr 15]. Available from http://www.cdc.gov/EID/content/13/4/06-0736.htm
12. 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.
13. Anders EM, Hartley CA, Jackson DC. Bovine and mouse serum beta inhibitors of influenza A viruses are mannose-binding lectins. Proc Natl Acad Sci U S A. 1990;87:4485–9. DOI: 10.1073/pnas.87.12.4485
14. World Organization for Animal Health. Avian influenza. In: Manual of diagnostic tests and vaccines for terrestrial animals, 5th ed. Updated Aug 7, 2005 [cited 2008 15 Apr]. Available from http://www.oie.int/eng/normes/mmanual/A_00037.htm

Address for correspondence: Martin Beer, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Suedufer 10, 17493 Greifswald-Insel Riems, Germany; email: martin.beer@fli.bund.de

Outbreak of Dengue and Chikungunya Fevers, Toamasina, Madagascar, 2006

Mahery Ratsitorahina,* Julie Harisoa,† Jocelyn Ratovonjato,* Sophie Biacabe,† Jean-Marc Reynes,* Hervé Zeller,‡ Yolande Raoelina,§ Antoine Talarmin,* Vincent Richard,* and Jean Louis Soares*

An outbreak of dengue-like syndrome occurred in Toamasina from January through March 2006. Dengue type I or chikungunya viruses were detected in 38 of 55 patients sampled. *Aedes albopictus* was the only potential vector collected. Of 4,242 randomly selected representative residents interviewed retrospectively, 67.5% reported a dengue-like syndrome during this period.

Arbovirus infections, arthropod-transmitted viral diseases, are common health risks in tropical and subtropical areas. Since early 2006, chikungunya fever, a crippling mosquito-borne disease, has emerged in the southwestern nations of the Indian Ocean. An epidemic started in Kenya in 2004 and in Moroni (Comoros Island) early in 2005 (*1*). Increasing incidence of the disease was first reported in April 2005 in the French island of Réunion. Approximately 5,000 cases were reported up to December, when a massive epidemic began (*2*). By the first week of March 2006, the virus had spread to the islands of Seychelles, Mauritius, and Mayotte (*1*).

During that same period, in January 2006, an outbreak of denguelike syndrome (DLS) was reported in Toamasina, on Madagascar's east coast. The results of the virologic and entomologic investigations are reported below. A retrospective cross-sectional study was also conducted to assess the extent of the outbreak.

The Study

Laboratory investigation began when the alert was given in January 2006. Serum samples were obtained from patients who had fever lasting ≤5 days and at least 3 of the following symptoms: headache, myalgia, arthralgia, retroorbital pain, or rash. Serum samples were sent from the health centers in Toamasina to the Institut Pasteur de Madagascar in Antananarivo in dry nitrogen containers and then stored at –80°C. Altogether, 55 patients were sampled; second samples were obtained from 14 patients. RNA extraction and chikungunya virus (CHIKV) detection were performed as previously described (*1*). Dengue virus (DENV) detection was performed according to the technique of Lanciotti et al. (*3*). Immunoglobulin (Ig) M and IgG against CHIKV and DENV were detected by ELISA as previously described (*4*).

Entomologic investigation was conducted February 14–17, 2006, to determine the abundance of DENV and CHIKV in urban mosquito vectors. Five neighborhoods in which human cases of DLS had been reported were explored. Mosquito larvae collected by manual method at all potential larvae breeding sites, inside and outside homes, were reared to obtain adults for species identification. The Breteau Index and Container Index were then calculated for the *Aedes* spp. identified. Collection of adult mosquitoes was performed indoors with pyrethrum spray catches and outside with US Centers for Disease Control and Prevention (CDC) light trap and oral aspirators (for hand catches).

Epidemiologic study was conducted March 21–28, 2006. A retrospective cross-sectional study was performed on Toamasina's population (≈200,000 inhabitants) by using a 2-stage cluster sampling (n = 378) at the neighborhood level. Every member of the household was interviewed with a standardized questionnaire based on personal history of sickness and travel. Data for children or those absent were given by present adults. Description of fever episodes and other clinical signs occurring after January 1, 2006, were collected. DLS is defined as a febrile illness and at least 2 other signs or symptoms: headache, joint pain, body pain, rash, and asthenia. Data were entered and analyzed by using EpiInfo (CDC, Atlanta, GA, USA).

DENV-1 or CHIKV was detected in 38 of 55 patients sampled. Co-infections were detected in 10 patients (Figure 1). Consequently, the outbreak was definitively attributed to DENV-1 and CHIKV.

Aedes albopictus was the unique DENV and CHIKV vector found. Only 6 adults of this mosquito species were caught. All of the positive larval breeding sites consisted of artificial containers (drums, buckets, coconut shells, discarded cans, tires, pots, and wet containers). Tires were the most important breeding sites identified (68%, n = 96). The Container and Breteau Indexes were 38.6 and 84.0, respectively. *Ae. albopictus* mosquito pools (n = 23) were tested by processing with CHIKV and DENV reverse transcription–PCRs. Five pools, including adults reared from larvae, were found to be CHIKV positive. However, no isolate was obtained from these pools by using AP61 cells.

*Institut Pasteur de Madagascar, Antananarivo, Madagascar; †Direction Provinciale de la Santé, Toamasina, Madagascar; ‡Institut Pasteur, Lyon, France ; and §Direction des Urgences et de la Lutte contre les Maladies Transmissibles, Antananarivo

DOI: 10.3201/eid1407.071521

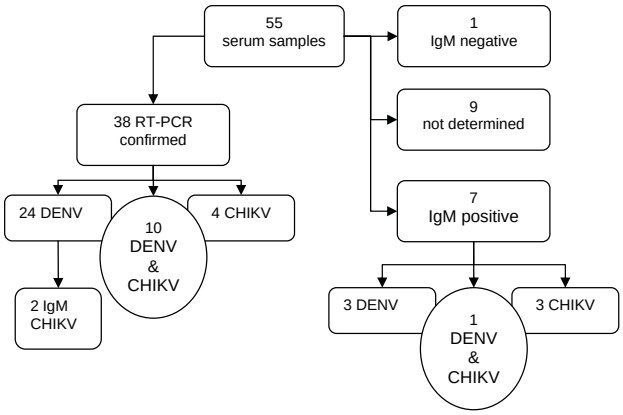


Figure 1. Laboratory diagnosis, dengue (DENV) and chikungunya (CHIKV) viruses, for 55 patients sampled, Madagascar, 2006. RT-PCR, reverse transcription–PCR; Ig, immunoglobulin.

A total of 4,242 residents (803 household units) in the 27 neighborhoods of Toamasina participated in the study. The mean age of participants was 24.9 years (95% confidence interval [CI] 24.4–25.4). The sex ratio (male:female) was 0.87. Most of the participants (n = 3,084; 72.7%) reported ≥1 previous episode of fever from January through March 2006. DLS was suspected in 2,863 (67.5%) residents, among whom 41 (1.4%) were hospitalized.

The estimated epidemic curve identified 2 outbreak periods. This distribution was emphasized by the report of a second episode of DLS by 110 patients. The peaks of the 2 periods were estimated in weeks 7 and 11 (Figure 2). The percentage of DLS in the studied population was high (40.2%–76.1%), regardless of age or sex. Rates of DLS did no differ in male and female patients. However, patients with DLS were older (mean age 26.8 years, 95% CI 26.2–27.4) than nonaffected persons (mean age 20.8 years, 95% CI 19.9–21.7; p<0.01). The most common features of the first episode of DLS (n = 110) were fever (100%), headache (96.4%), joint pain (79.1%), asthenia (76.4%), myalgia (76.4%), pruritus (40.0%), and rash (13.6%). When these 110 patients exhibited a second episode of DLS, the most common clinical features were fever (100%), headache (90.0%), myalgia (73.6%), joint pain (68.2%), and pruritus (28.2%).

Conclusions

An outbreak caused by both CHIKV and DENV-1 occurred in Madagascar. The notification of CHIKV in the island occurred during a large epidemic that hit the southwestern islands of the Indian Ocean. The Malagasy CHIKV E1 partial nucleotide sequences were similar to those of the Indian Ocean outbreak (Réunion, Seychelle, Mayotte, Mauritius islands) and represented a distinct clade within the large east-central and South African phylogroup (*1*). DENV-1 was also circulating during this outbreak in the eastern part of the island. A few DENV-1 isolates were already obtained from patients visiting the northwestern coast of the island in early 2005. Molecular characterization of these isolates showed that they were similar to DENV-1 strains circulating in 2004 in Réunion and indicated their regional origin (H. Zeller, pers. comm.).

Ae. albopictus was the only urban vector of DENV or CHIKV detected during this outbreak. This species had already been reported in the eastern coast of Madagascar (*5*). Individual mosquitoes from this species, sampled in Antananarivo (in the highlands of Madagascar) in the 1970s and more recently in northern Madagascar, are highly susceptible to CHIKV and DENV (*6–8*). This situation, associated with a high larvae index and probably a nonimmune population, led to a high rate of transmission, as indicated by the high percentage of DENV-1 and CHIKV co-infection detected in the 55 patients tested (18%) and the high attack rate (67.5%).

The cocirculation of DENV-1 and CHIKV during the period may explain the 2 peaks observed in the epidemic curve. However, because the study was based on clinical observations alone, we could not measure the relative contribution for each peak. All age groups were affected. This result is likely due in part to the recent emergence of these viruses in Madagascar. However, children and particularly those <1 year of age might be less exposed to *Ae. albopictus* because they are often indoors and are fully covered. Underreporting could also be an explanation for this moderate prevalence in children.

During this epidemic, vector control measures (removal of tires from rooftops, information campaign, education of the community) were undertaken by the local authorities. After the outbreak, the Malagasy Ministry of Health

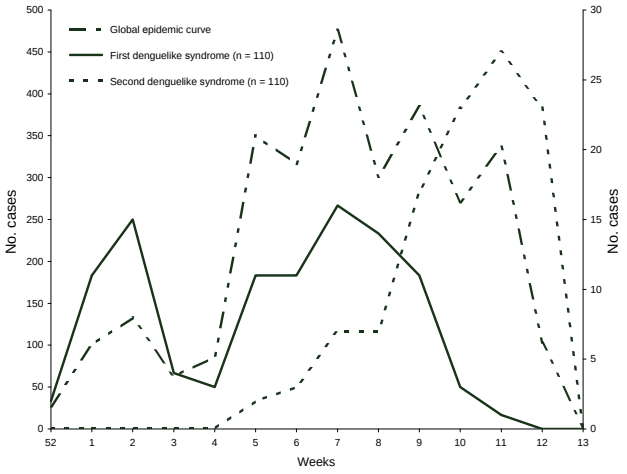


Figure 2. Outbreak curve of dengue and chikungunya fevers in Toamasina, January 1–March 28, 2006.

implemented a sentinel surveillance system to monitor and control epidemic arboviral diseases. However, the emergence of this regional outbreak also demonstrates the need for coordinating surveillance systems in the islands in the southwestern Indian Ocean.

This work received financial support from the Institut Pasteur de Madagascar.

Dr Ratsitorahina is a physician working in the Epidemiology Unit at the Institut Pasteur de Madagascar. He has conducted epidemiologic research in Madagascar for >10 years.

References

1. Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med. 2006;3:e263. Epub 2006 May 23. DOI: 10.1371/journal.pmed.0030263
2. Renault P, Solet JL, Sissoko D, Balleydier E, Larrieu S, Filleul L, et al. A major epidemic of chikungunya virus infection on Reunion Island, France, 2005–2006. Am J Trop Med Hyg. 2007;77:727–31.
3. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol. 1992;30:545–51.
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. Emerg Infect Dis. 2001;7:692–6.
5. Fontenille D, Rodhain F. Biology and distribution of *Aedes albopictus* and *Aedes aegypti* in Madagascar. J Am Mosq Control Assoc. 1989;5:219–25.
6. Gubler DJ, Rosen L. Variation among geographic strains of *Aedes albopictus* in susceptibility to infection with dengue viruses. Am J Trop Med Hyg. 1976;25:318–25.
7. Tesh RB, Gubler DJ, Rosen L. Variation among goegraphic strains of *Aedes albopictus* in susceptibility to infection with chikungunya virus. Am J Trop Med Hyg. 1976;25:326–35.
8. Vazeille M, Mousson L, Rakatoarivony I, Villeret R, Rodhain F, Duchemin JB, et al. Population genetic structure and competence as a vector for dengue type 2 virus of *Aedes aegypti* and *Aedes albopictus* from Madagascar. Am J Trop Med Hyg. 2001;65:491–7.

Address for correspondence: Vincent Richard, Unité d'Epidémiologie, Institut Pasteur de Madagascar, BP 1274, Antananarivo 101, Madagascar; email: vrichard@pasteur.mg

Integrating Host Genomics with Surveillance for Invasive Bacterial Diseases

Dana C. Crawford,* Shanta M. Zimmer,† Craig A. Morin,‡ Nancy E. Messonnier,§ Ruth Lynfield,‡ Qian Yi,¶ Cynthia Shephard,¶ Michelle Wong,¶ Mark J. Rieder,¶ Robert J. Livingston,¶ Deborah A. Nickerson,¶ Cynthia G. Whitney,§ and Jairam Lingappa¶

We tested the feasibility of linking Active Bacterial Core surveillance, a prospective, population-based surveillance system for invasive bacterial disease, to a newborn dried blood spot (nDBS) repository. Using nDBS specimens, we resequenced CD46, putative host gene receptor for *Neisseria meningitidis*, and identified variants associated with susceptibility to this disease.

Host genetic factors may help predict susceptibility to infectious diseases and could target high-risk populations for public health interventions such as vaccination. However, even with cost-effective genotyping technologies (*1*), small cohorts and limited associated epidemiologic data may lead to underpowered studies. Existing large population-based surveillance systems, if integrated with appropriate genetic material, could contribute crucial hypotheses and generate data to identify host factors underlying infectious diseases.

Active Bacterial Core surveillance (ABCs) is a network of state health departments, academic institutions, and local collaborators funded by the Centers for Disease Control and Prevention (CDC). This network conducts population-based surveillance for invasive bacterial pathogens, including encapsulated bacteria *Haemophilus influenzae*, *Neisseria meningitides*, and *Streptococcus pneumoniae* (*2*); the Minnesota Department of Health has been involved in ABCs since 1995. Use of ABCs data to identify potential genetic risk factors could identify high-risk groups for vaccination with conjugated polysaccharide vaccines targeted against encapsulated bacterial pathogens. In particular, *N. meningitidis,* the causative agent for meningococcal disease, has a baseline carriage rate of 5%–10% (*3*), a US incidence of 1 case/100,000 persons (*2*,*4*), and a 10%–15% case-fatality rate (*2*). Given the epidemiology of *N. meningitidis* and recent data suggesting a high sibling risk ratio (*5*), it is plausible that host factors (*6*) modify susceptibility or severity to meningococcal disease.

*Vanderbilt University, Nashville, Tennessee, USA; †Emory University, Atlanta, Georgia, USA; ‡Minnesota Department of Health, St. Paul, Minnesota, USA; §Centers for Disease Control and Prevention, Atlanta; and ¶University of Washington, Seattle, Washington, USA

DOI: 10.3201/eid1407.071287

The Study

Although ABCs provides a unique epidemiologic context for assessing host genetic risk factors for *N. meningitidis*, host DNA is not collected. However, genetic material is collected prospectively from all infants through state-based newborn dried blood spot (nDBS) programs (*7*). We cross-referenced ABCs data to the state's nDBS repository to identify nDBSs from Minnesota ABCs case-patients and controls.

ABCs data were evaluated to identify cases of invasive encapsulated bacterial infection (*H. influenzae*, *N. meningitides*, or *S. pneumoniae*) in persons born January 1, 1997, through December 31, 2000. Parents or guardians of case-patients were contacted by mail for written consent (and where needed, childhood consent). ABCs data from case-patients with parental consent and from case-patients who did not respond after 2 successive mailings were included in the study. Two controls, selected from among children with nDBSs, were matched per case by date of birth, race, and hospital of birth. ABCs data and case and control nDBSs were stripped of linkage to personal identifiers.

Human subject review and approval was obtained through CDC and the Minnesota Department of Health before study initiation. Once ABCs data and nDBSs were deidentified, the CDC institutional review board closed the project, which enabled genomic studies with unidentifiable nDBS specimens. The University of Washington human subjects division subsequently granted a certificate of exemption.

We identified 486 cases of invasive disease: 22 with *N. meningitidis*, 19 with *H. influenzae*, and 445 with *S. pneumoniae*. One case-patient refused consent and was dropped from the study; 88 case-patients (18.1%) gave written consent, and 397 (81.7%) did not respond after 2 mailings. The nDBSs were identified for 406 (84%) case-patients. Among controls, 812 (100%) were matched to case-patients by date of birth and race, and 674 (83%) were matched by date of birth, race, and hospital of birth. A total of 22 *N. menigitidis* case-patients and 44 controls with nDBSs defined the case–control (CC) study. Case-patient characteristics are shown in Table 1. No deaths were documented among the ABCs case-patients.

Genomic DNA was amplified from 3-mm punches of 1/2″ nDBSs by using multiple displacement techniques (*8*)

Table 1. Characteristics of 22 case-patients infected with *Neisseria meningitidis*

Characteristic	Value
Female, no. (%)	11 (50.0)
Race-ethnicity, no. (%)	
White	16 (72.7)
Black	3 (13.6)
Asian	2 (9.1)
Other	1 (4.6)
Mean age, d (range)	144 (9 d–3.4 y)
Bacteremia with focus, no. (%)	12 (54.6)
Meningitis, no. (%)	10 (45.5)
Serogroup, no. (%)	
B	10 (45.5)
C	5 (22.7)
Y	5 (22.7)
W135	1 (4.5)
Not groupable	1 (4.5)

(Molecular Staging, Inc., New Haven, CT, USA). We resequenced the *CD46* gene (*9*), a putative host gene receptor for *N. meningitidis* (*10*,*11*), in 143 samples from 66 CC study samples and 77 Coriell Cell Repository (CCR; Camden, NJ, USA) samples (online Technical Appendix, available from www.cdc.gov/EID/content/14/7/1138-Techapp.pdf) (GenBank accession no. AY916779). Standard dye primer and termination sequencing with sequence assembly and polymorphism discovery was performed through the Program for Genomic Applications (National Heart, Lung, and Blood Institute, Bethesda, MD, USA) (Seattle SNPs [single nucleotide polymorphisms]) (*12*). Of 269 diallelic sites (SNPs), 173 (64%) were in the CC study samples and 59 (34%) were unique to the CC study samples (Table 2, online Technical Appendix). Hardy-Weinberg equilibrium (HWE) was used to evaluate genotyping errors; most SNPs in CCR (97.6%) and CC study samples (96.5%) samples met HWE ($p \geq 0.05$).

The overall genotyping call rate for nDBS CC study samples was 89.5% compared with 96.7% for cell line–derived CCR DNAs ($p < 0.0001$, by χ^2 test). Among CC study samples, 62% had highly useable DNA quality as assigned by MSI after amplification. The DNA quality rating predicted genotyping call rate (generalized linear model $R^2 = 0.52$, $p < 0.0001$) with highly useable samples having a call rate of 93.9%.

Among 173 SNPs in the CC study samples, 116 (67%) were in case-patients, 146 (84.3%) in controls, and 89 (51.15%) in both groups (Table 2). We grouped SNPs (minor allele frequency >5%) from the European-American CCR samples into bins on the basis of linkage disequilibrium ($r^2 > 0.80$) by using the LDSelect algorithm (*13*). Among 17 CD46 tagSNPs tested (each representing 1 bin), site 6420 (rs41317049) was significantly associated with meningococcal disease (by Fisher exact test) assuming a general genotype model (separately comparing homozygous major, heterozygous, and homozygous minor alleles; $p = 0.0176$) and a dominant genetic model (homozygous major allele vs. all others; $p = 0.0440$) (online Technical Appendix). Logistic regression showed that, adjusting for age and sex, SNP 6420 had borderline significance ($p = 0.051$), with increased odds of disease (odds ratio 4.38) for GT/GG versus TT genotypes (95% confidence interval 0.99–19.30). Given a sample size of 16 case-patients and 32 controls, a general genotype model is powered ($\alpha = 0.05$, $\beta = 0.80$) to detect an odds ratio from 3.6 through 6.6, depending on the minor allele frequency of the risk-conferring SNP.

Conclusions

We integrated an active, population-based, prospective disease surveillance system post hoc with a population-based, prospective nDBS repository to combine disease surveillance information with genetic specimens. Although nDBSs have been used to establish prevalence (*14*), nDBSs have not been linked post hoc to an extensive clinical/epidemiologic database for genetic hypothesis generation.

To test use of these nDBS specimens for hypothesis generation, we resequenced a potential meningococcal risk factor, the putative meningogoccal receptor CD46, for genetic variation discovery. Highly useable samples had genotyping call rates similar to those of cell-line extracted CCR DNA (94% vs. 97%). Furthermore, on the basis of

Table 2. Number of diallelic sites (SNPs) identified for CD46, by population*

Population	Sample size	No. SNPs† (population-specific SNPs)‡	No. common SNPs§
European American	23	93 (32)	58
African	24	130 (74)¶	68
Asian	24	88 (30)	46
Hispanic	6	56 (3)	56
Study samples	66	173 (59)	66
Case-patients	22	116 (27)	70
Controls	44	146 (57)	

*SNPs, single nucleotide polymorphisms.
†Includes SNPs and diallelic insertion/deletion polymorphisms (indels).
‡No. SNPs identified in only that racial/ethnic population.
§Common SNPs defined as having a minor allele frequency >5%.
¶Two SNPs (sites 18924 and 28122) were specific to the African cohort but in regions not resequenced sufficiently in other populations. Additional genotyping is needed to conclusively identify these as African specific.

HWE and similar allele frequencies between the CC study samples and CCR samples, we did not detect heterozygote bias. Ongoing studies are evaluating use of other technologies to genotype these samples.

We identified an association between an SNP (6420; rs41317049) in the candidate gene CD46 and case status for *N. meningitidis*. The intronic location of SNP 6420 and existence of CD46 splicing isoforms (*15*) suggest a possible role of altered splicing. However, the genetic association itself and any hypothesized mechanism require future replication studies to rule out alternative explanations of chance, population stratification, causality/susceptibility, or linkage disequilibrium.

Our results are novel, but this pilot study was powered for large genetic effects. Furthermore, the cohort was primarily of European descent, and results were not adjusted for multiple comparisons. Given the surveillance target period and duration that Minnesota retained nDBS specimens, our study cohort was children <5 years of age, the age range targeted for conjugate polysaccharide vaccines. With the growing importance of using nDBSs for genetic studies (*7*), future studies should assess whether this approach is generalizable. Use of existing large, surveillance databases linked to nDBS repositories will facilitate replication of the genetic association specifically, and more generally, evaluation of host genomics of susceptibility to infectious diseases.

Acknowledgments

We thank Janet McNicholl and David Stephens for inspiring the effort to link ABCs and nDBS, ABCs staff at CDC for providing invaluable support throughout the project, and Robin Hampton for technical assistance in managing and organizing blood spots and whole genome amplified DNA.

The resequencing work reported was supported by grants from the National Heart, Lung, and Blood Institute's Program for Genomic Applications (U01 HL66682 and U01 HL66728) and the National Institute of Environmental Health Science's Environmental Genome Project (N01 ES15478). Support to develop this cohort was also provided by the CDC National Center for Infectious Disease Genetics Working Group.

Dr Crawford is an assistant professor at the Center for Human Genetics Research at Vanderbilt University. Her research interests include the broad area of identifying genetic variations associated with complex human diseases or traits ranging from cardiovascular quantitative traits to infectious diseases.

References

1. Poland H. Integrating genomics against infectious disease. Nat Genet. 2006;38:513–4. DOI: 10.1038/ng0506-513
2. Schuchat A, Hilger T, Zell E, Farley MM, Reingold A, Harrison L, et al. Active bacterial core surveillance of the emerging infections program network. Emerg Infect Dis. 2001;7:92–9.
3. Greenfield S, Sheehe PR, Feldman HA. Meningococcal carriage in a population of "normal" families. J Infect Dis. 1971;123:67–73.
4. Rosenstein NE, Perkins BA, Stephens DS, Lefkowitz L, Cartter ML, Danila R, et al. The changing epidemiology of meningococcal disease in the United States, 1992–1996. J Infect Dis. 1999;180:1894–901. DOI: 10.1086/315158
5. Haralambous E, Weiss HA, Radalowicz A, Hibberd ML, Booy R, Levin M. Sibling familial risk ratio of meningococcal disease in UK Caucasians. Epidemiol Infect. 2003;130:413–8.
6. Dahmer MK, Randolph A, Vitali S, Quasney MW. Genetic polymorphisms in sepsis. Pediatr Crit Care Med. 2005;6:S61–73. DOI: 10.1097/01.PCC.0000161970.44470.C7
7. Olney RS, Moore CA, Ojodu JA, Lindegren ML, Hannon WH. Storage and use of residual dried blood spots from state newborn screening programs. J Pediatr. 2006;148:618–22. DOI: 10.1016/j.jpeds.2005.12.053
8. Lovmar L, Syvanen A-C. Multiple displacement amplification to create a long-lasting source of DNA for genetic studies. Hum Mutat. 2006;27:603–14. DOI: 10.1002/humu.20341
9. Seya T, Hirano A, Matsumoto M, Nomura M, Ueda S. Human membrane cofactor protein (MCP, CD46): multiple isoforms and functions. Int J Biochem Cell Biol. 1999;31:1255–60. DOI: 10.1016/S1357-2725(99)00092-8
10. Kallstrom H, Liszewski MK, Atkinson JP, Jonsson AB. Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria.* Mol Microbiol. 1997;25:639–47. DOI: 10.1046/j.1365-2958.1997.4841857.x
11. Johansson L, Rytkonen A, Bergman P, Albiger B, Kallstrom H, Hokfelt T, et al. CD46 in meningococcal disease. Science. 2003;301:373–5. DOI: 10.1126/science.1086476
12. Crawford DC, Akey DT, Nickerson DA. The patterns of natural variation in human genes. Annu Rev Genomics Hum Genet. 2005;6:287–312. DOI: 10.1146/annurev.genom.6.080604.162309
13. Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. Am J Hum Genet. 2004;74:106–20. DOI: 10.1086/381000
14. Steinberg K, Beck J, Nickerson D, Garcia-Closas M, Gallagher M, Caggana M, et al. DNA banking for epidemiologic studies: a review of current practices. Epidemiology. 2002;13:246–54. DOI: 10.1097/00001648-200205000-00003
15. Dhiman N, Jacobson RM, Poland GA. Measles virus receptors: SLAM and CD46. Rev Med Virol. 2004;14:217–29. DOI: 10.1002/rmv.430

Address for correspondence: Jairam Lingappa, Department of Medicine, University of Washington, Box 359927, Seattle, WA 98195-9927, USA; email: lingappa@u.washington.edu

Invasive Amebiasis in Men Who Have Sex with Men, Australia

Damien Stark,* Sebastian J. van Hal,* Gail Matthews,† John Harkness,* and Deborah Marriott*

Entamoeba histolytica is a pathogenic ameba that has recently been recognized as an emerging pathogen in men who have sex with men (MSM) in Asia-Pacific countries where it is not endemic, i.e., Japan, Taiwan, and Republic of Korea. We report locally acquired invasive amebiasis in Sydney, Australia, exclusively in MSM.

Entamoeba histolytica is a pathogenic ameba that can cause invasive intestinal and extra-intestinal disease. The most frequent manifestations of invasive amebiasis are colitis and liver abscesses (*1*). Although *E. histolytica* is one of the most common parasitic infections worldwide, invasive disease remains uncommon in industrialized counties. Recent studies from Japan, Taiwan, and Republic of Korea, areas where *E. histolytica* endemicity is generally low, suggest that amebiasis is an emerging parasitic infection that occurs exclusively in men who have sex with men (MSM) (*2*–*6*). In Australia, the documented incidence of *Entamoeba* spp. in the general population is 1%–4% (*1*). In MSM, the rates of *Entamoeba* spp. carriage were previously documented to be as high as 37% (*7*). However, these studies failed to differentiate the pathogenic *E. histolytica* from the morphologically identical nonpathogenic *E. moshkovskii* and *E. dispar*, therefore leaving the accuracy of these results in question. A study in Australia that used molecular methods showed *E. histolytica* prevalence rates in MSM to be as low as 0.1% (*8*). Impaired host immunity is associated with increased pathogenicity of invasive amebiasis. Recent studies indicate an increased risk for invasive amebiasis among persons with HIV (*9*,*10*). We report 5 cases of invasive amebiasis in MSM from Sydney, New South Wales, Australia, from December 2006 through October 2007.

The Cases

Of the 5 patients, 3 had amebic colitis and 2 had amebic liver abscesses, all were MSM, 4 were HIV-infected, mean age was 45 years (range 35–57 years), and median CD4 count was 713 cells/mm^3 (Table). No associations among any of the patients were noted. All patients were receiving highly active antiretroviral therapy at the time of examination. Of the 5 patients, 4 had not traveled extensively in the past 4 years, but the remaining patient had traveled to Malaysia and China 6 months before onset of colitis. Other possible risk factors for acquisition were identified in 4 patients: 3 had had high-risk sex behavior at sex-on-premises venues, and the other had had a male partner who had traveled to countries where invasive amebiasis was highly endemic.

The patients had bloody diarrhea and abdominal pain for 2–4 weeks. Routine fecal cultures were negative for bacterial pathogens. Microscopic examination of permanently fixed, stained fecal smears was positive for *E. histolytica*/*dispar/moshkovskii* complex. Diagnosis of *E. histolytica* was confirmed by PCR targeting the small subunit ribosomal DNA as described (*8*). Results of serologic examination of these patients were positive; all titers were >256 according to indirect hemagglutination antibody assay, which confirmed invasive disease. However, although antibodies against amebae indicate invasive disease, these antibodies can also be seen in persons with asymptomatic colonization with amebae (*1*).

The 2 patients with liver abscesses each had large, solitary abscesses in the right lobe of the liver (8 × 6 × 6 cm and 7.5 × 6.2 × 6.6 cm). In 1 patient, the abscess ruptured through the liver capsule, and collapse of the right middle and lower lung lobes and a resultant pleural effusion complicated the subphrenic collection of pus from the abscess. Levels of liver enzymes (alkaline phosphatase and gamma glutamyl transferase), C-reactive protein, and neutrophils (absolute numbers) were raised. Both patients underwent percutaneous drainage of their liver abscess; cultures of aspirated pus were negative for bacteria and fungi. Results of indirect hemagglutination antibody assay were positive; titer was ≥256. Microscopy and PCR of fecal samples were negative for *E. histolytica.* For these 2 patients with amebic liver abscess, the diagnosis was delayed; they had had symptoms for >2 weeks and were then treated for bacterial liver abscess before the correct diagnosis was made.

All 5 patients made a successful recovery after treatment with metronidazole. In addition, all were treated for cyst carriage with paromomycin, a luminal amebicide.

Conclusions

E. histolytica carriage and invasive disease are common in the Asia-Pacific region, especially in developing countries. In countries where *E. histolytica* prevalence is low, such as Japan, Taiwan, Republic of Korea, and Australia, rates of amebiasis are low and invasive amebiasis is uncommon. Recent reports from a number of these coun-

*St. Vincent's Hospital, Darlinghurst, New South Wales, Australia; and †University of New South Wales, Sydney, New South Wales, Australia

DOI: 10.3201/eid1407.080017

Table. Summary of clinical characteristics of 5 patients with invasive amebiasis, Australia, December 2006–October 2007*

Age, y	HIV status	CD4 count, cells/mm^3	HIV viral load, copies/mL	Clinical condition	Identified risk factors
35	+	672	ND	Colitis	SOP
42	+	756	<40	Colitis	SOP and travel in past 6 mo
43	+	754	ND	Colitis	SOP
57	–	ND	ND	Liver abscess	Extensive travel by ex-partner in past 8 mo
52	+	256	30,700	Liver abscess	None

*ND, not done; SOP, visited sex-on-premises venue.

tries, however, suggest that invasive amebiasis is emerging as an increasingly common infection, specifically in the MSM population (*2*–*6*). MSM have a higher risk than others for intestinal parasite carriage; not only are they substantially more likely to harbor intestinal protozoa, but they are also more likely to harbor multiple parasites (*11*). These protozoa are transmitted by the fecal–oral route; high rates of oral–anal sex by MSM are considered the reason for increased rates of carriage. Because *E. histolytica* is also transmitted by the fecal–oral route, MSM may also have an increased risk for *E. histolytica* carriage. This higher rate of asymptomatic carriage is likely to translate into a greater risk for invasive disease. Recent seroprevalence studies in Taiwan that used indirect hemagglutination antibody assay have confirmed MSM's statistically significant higher risk for *E. histolytica* exposure (*5*).

In Japan, amebiasis has become endemic in MSM; symptomatic *E. histolytica* infection occurs almost exclusively in middle-aged MSM in the large cities of Japan (*2*,*3*). Similar findings are reported for MSM in Taiwan (*4*,*5*). More recently, a study from the Republic of Korea documented invasive amebiasis (amebic liver abscess) in HIV-infected MSM (*6*). To date, the emergence of *E. histolytica* infections in MSM seems to be limited to the Asia-Pacific region. In a large retrospective study of 34,000 HIV-infected patients in United States, only 2 patients had invasive amebiasis (*12*). The reasons for this geographic variation are unclear, but it is likely linked to the higher background prevalence of *E. histolytica* infections in Asia. Regional *E. histolytica* strains show a high degree of diversity but no major differences between regional genotypes; other factors relating to host or virulence factors may be important but are as yet undetermined (*3*). We now report local acquisition of *E. histolytica* by MSM in Australia; as shown by the above 5 cases of invasive disease and 3 previous cases of noninvasive infections also acquired locally (*13*).

Of note, 4 of the 5 patients we report were HIV infected. Seroprevalence rates of *E. histolytica* (determined by indirect hemagglutination antibody assay) are higher for HIV-infected persons then for HIV-nonninfected persons, although the reasons are unclear (*5*,*14*). Higher rates of *E. histolytica* carriage in MSM likely reflect high-risk sex behavior and multiple exposures, resulting in increased risk for acquisition. This hypothesis is supported by the high rates of sexually transmitted infections that occur in Australian MSM who visit sex-on-premises venues (*15*). Antibody responses predominantly occur with invasive disease. Whether immunosuppression caused by HIV infection attenuates the risk for invasive amebiasis is unknown. Historically, the evidence has been contradictory, and most published studies had had severe limitations. Nevertheless, more recent data seem to indicate that HIV-infected persons are at increased risk for invasive amebiasis (*9*,*10*).

The emergence of *E. histolytica* in MSM is of public health concern because it has the potential to become endemic in this population in Australia and to cause severe disease. Further study is needed to identify the reasons for the geographic variation and the role of *E. histolytica* in invasive disease. In conclusion, invasive amebiasis has the potential to emerge as an important parasitic infection in the Asia-Pacific region, especially in HIV-infected MSM in countries where *E. histolytica* is not endemic.

Dr Stark is a senior hospital scientist at St. Vincent's Hospital, Darlinghurst. His research interests are directed at molecular diagnosis of parasitic diseases.

References

1. van Hal SJ, Stark DJ, Fotedar R, Marriott D, Ellis JT, Harkness JL. Amoebiasis: current status in Australia. Med J Aust. 2007;186:412–6.
2. Ohnishi K, Murata M. Present characteristics of symptomatic amebiasis due to *Entamoeba histolytica* in the east-southeast area of Tokyo. Epidemiol Infect. 1997;119:363–7. DOI: 10.1017/S0950268897008236
3. Ohnishi K, Kato Y, Imamura A, Fukayama M, Tsunoda T, Sakaue Y, et al. Present characteristics of symptomatic *Entamoeba histolytica* infection in the big cities of Japan. Epidemiol Infect. 2004;132:57–60. DOI: 10.1017/S0950268803001389
4. Hung CC, Deng HY, Hsiao WH, Hsieh SM, Hsiao CF, Chen MY, et al. Invasive amebiasis as an emerging parasitic disease in patients with human immunodeficiency virus type 1 infection in Taiwan. Arch Intern Med. 2005;165:409–15. DOI: 10.1001/archinte.165.4.409
5. Tsai JJ, Sun HY, Ke LY, Tsai KS, Chang SY, Hsieh SM, et al. Higher seroprevalence of *Entamoeba histolytica* infection is associated with human immunodeficiency virus type 1 infection in Taiwan. Am J Trop Med Hyg. 2006;74:1016–9.
6. Park WB, Choe PG, Jo JH, Kim SH, Bang JH, Kim HB, et al. Amebic liver abscess in HIV-infected patients, Republic of Korea. Emerg Infect Dis. 2007;13:516–7.

7. Law CL, Walker J, Qassim MH. Factors associated with the detection of *Entamoeba histolytica* in homosexual men. Int J STD AIDS. 1991;2:346–50.
8. Fotedar R, Stark D, Beebe N, Marriott D, Ellis J, Harkness J. PCR detection of *Entamoeba histolytica, Entamoeba dispar*, and *Entamoeba moshkovskii* in stool samples from Sydney, Australia. J Clin Microbiol. 2007;45:1035–7. DOI: 10.1128/JCM.02144-06
9. Seeto RK, Rockey DC. Amebic liver abscess: epidemiology, clinical features, and outcome. West J Med. 1999;170:104–9.
10. Hung CC, Ji DD, Sun HY, Lee YT, Hsu SY, Chang SY, et al. Increased risk for *Entamoeba histolytica* infection and invasive amebiasis in HIV-seropositive men who have sex with men in Taiwan. PLoS Negl Trop Dis. 2008;2:e175. DOI: 10.1371/journal.pntd.0000175
11. Stark D, Fotedar R, van Hal S, Beebe N, Marriott D, Ellis JT, et al. Prevalence of enteric protozoa in human immunodeficiency virus (HIV)–positive and HIV-negative men who have sex with men from Sydney, Australia. Am J Trop Med Hyg. 2007;76:549–52.
12. Lowther SA, Dworkin MS, Hanson DL. *Entamoeba histolytica/Entamoeba dispar* infections in human immunodeficiency virus–-infected patients in the United States. Clin Infect Dis. 2000;30:955–9. DOI: 10.1086/313811
13. Stark DJ, Fotedar R, Ellis JT, Harkness JL. Locally acquired infection with *Entamoeba histolytica* in men who have sex with men in Australia. Med J Aust. 2006;185:417.
14. Ko NY, Lee HC, Chang JL, Lee NY, Chang CM, Lee MP, et al. Prevalence of human immunodeficiency virus and sexually transmitted infections and risky sexual behaviors among men visiting gay bathhouses in Taiwan. Sex Transm Dis. 2006;33:467–73. DOI: 10.1097/01.olq.0000204512.15297.5f
15. O'Sullivan B, Delpech V, Pontivivo G, Karagiannis T, Marriott D, Harkness J, et al. Shigellosis linked to sex venues, Australia. Emerg Infect Dis. 2002;8:862–4.

Address for correspondence: Damien Stark, Division of Microbiology, SydPath, St. Vincent's Hospital, Darlinghurst 2010, NSW, Australia; email: dstark@stvincents.com.au

Persistence of Diphtheria, Hyderabad, India, 2003–2006

Sailaja Bitragunta,* Manoj V. Murhekar,* Yvan J. Hutin,* Padmanabha P. Penumur,† and Mohan D. Gupte*

During 2003–2006, diphtheria rates in Hyderabad, India, were higher among persons 5–19 years of age, women, and Muslims than among other groups. Vaccine was efficacious among those who received ≥4 doses. The proportion of the population receiving boosters was low, especially among Muslims. We recommend increasing booster dose coverage.

Diphtheria is a disease caused by the exotoxin produced by *Corynebacterium diphtheriae.* The Expanded Programme of Immunization of the World Health Organization recommends 3 doses of the diphtheria, pertussis, and tetanus (DPT) vaccine starting at 6 weeks of age with additional doses of diphtheria vaccine in countries where resources permit (*1*). Many national immunization programs, including the Universal Immunization Programme of India, offer 2 booster doses at 18 months and between 54 and 72 months of age. After 3 doses of primary vaccine, protective levels of antitoxin develop in 94% to 100% of children (*1*,*2*). However, without booster doses, over time toxoid-induced antibody drops below protective levels (*2*,*3*).

In 2005, India contributed 5,826 (71%) of the 8,229 diphtheria cases reported globally (*4*). Of the total cases from India, 4,161 (71%) were from the state of Andhra Pradesh (*5*). Hyderabad, the state capital, contributed 663 (16%) of the total cases from the state (Government of Andhra Pradesh, unpub. data). The administrative coverage of primary vaccination among children 12–23 months of age (a performance indicator for Universal Immunization Programme [UIP]) ranged from 98% to 100% in the city from 1995 through 2006. We conducted a study to 1) describe the epidemiology of diphtheria in terms of time, place, and person; 2) estimate vaccine coverage; and 3) estimate diphtheria vaccine efficacy.

*National Institute of Epidemiology, Chennai, India; and †Fever Hospital, Hyderabad, India

DOI: 10.3201/eid1407.071167

The Study

Diphtheria patients identified in Hyderabad and neighboring districts are admitted to the Fever Hospital. Case-patients undergo a smear examination of the characteristic patch of thick gray membrane and samples are cultured for *C. diphtheriae*. To describe the epidemiology of diphtheria, we included cases defined as an acute febrile infection with gray-white patch in pharynx, tonsils, or fauces among residents of Hyderabad admitted to Fever Hospital during 2003–2006. We obtained data regarding age, sex, religion, month and year of occurrence, and circle (municipal administrative subdivision) of residence from the medical records of 2,685 diphtheria case-patients admitted during 2003–2006. Thirty-one case-patients died (overall case-fatality rate 1.2%). Diphtheria occurred throughout the year with lower incidences during July and August (Figure). Annual incidence increased from 11/100,000 to 23/100,000 from 2003 through 2006 (χ^2 trend 152; p = 0.00001). Median age of case-patients was 17 years (range 9 months–80 years). Attack rates were lowest among infants, increased with age, and reached a maximum among children 10–14 years of age. Rates were higher among girls and women (Table 1). Of the 2,685 case-patients, 70% were Muslim, who had rates 3 times higher than other communities. Circle-specific attack rates ranged from 17/100,000 to 25/100,000 and were highest in the first 4 circles of the city where a predominantly Muslim population resided; this area accounted for 90% of cases in 2003–2006. During 2006, 81% of the cases were either smear or culture positive.

We surveyed the 7 circles of the city to estimate primary vaccination coverage among children 12–23 months of age, fourth diphtheria dose (DPT) among those 18–36 months of age, and fifth diphtheria dose (DT) among children 54–72 months of age, respectively. We selected a stratified sample of 658 children in each age group. Criteria for a completely vaccinated child were defined according to the UIP vaccination schedule by age group.

Coverage for primary vaccination, fourth, and fifth doses was 90% (95% confidence interval [CI] 89%–90%),

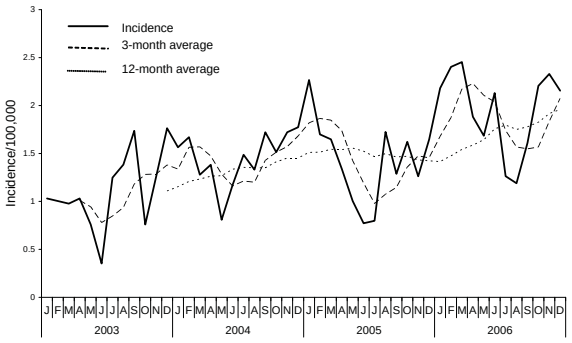


Figure. Incidence of diphtheria in Hyderabad, India, 2003–2006.

Table 1. Average yearly attack rate of diphtheria by age and sex, Hyderabad, India, 2003–2006

Demographic characteristics	No. cases, 2003–2006	Average population, 2003–2006	Annual attack rate/100,000 population
Age, y			
≤1	8	81,050	2
2–4	116	240,450	12
5–9	455	415,673	27
10–14	530	455,426	29
15–19	431	450,408	24
20–44	1,054	1,583,569	17
≥45	91	632,964	4
Sex			
Male	1,153	1,983,960	15
Female	1,532	1,875,580	20
Religion			
Non-Muslim	811	2,270,695	9
Muslim	1,874	1,588,845	29
Total	2,685	3,859,540	17

60% (95% CI 59%–60%), and 33% (95% CI 33%–34%), respectively. Although coverage for primary vaccination did not differ among Muslims and non-Muslims (coverage ratio 0.95, 95% CI, 0.90%–1.1%), coverage for fourth and fifth doses was lower among Muslims (coverage ratios 0.86, 95% CI 0.75%–0.99% and 0.59, 95% CI 0.5%–0.8%, respectively).

We compared laboratory-confirmed case-patients ≤10 years of age who lived in Hyderabad with age- and residence-matched controls. Information about educational status of parents, monthly family income, religion, and number of vaccine doses received was collected through interviews of mothers or guardians. Vaccination status was ascertained from vaccination cards or the mother's history when a card was not available. All exposures were included in a stepwise conditional logistic regression by using Epi Info (Centers for Disease Control and Prevention, Atlanta, GA, USA). Vaccine efficacy (%) was calculated by using the 1 – odds ratio formula (*6*). We included all children in the analysis to estimate vaccine efficacy for the first 4 doses. However, to estimate the efficacy of 5 doses of diphtheria vaccine, we restricted the analysis to children 5–10 years of age because the fifth dose of the vaccine is given to children >4.5 years of age.

We included 123 case-patients in the case-control study. Only 20 (16%) controls and 23 (19%) case-patients had a vaccination card. The median age of case-patients was 7 years and 50% were girls. Twenty-one children (17%) were younger than 5 years of age. When adjusted for religion, family income, and literacy status of parents, vaccine efficacy increased from 49% (95% CI 0%–80%) for 3 doses to 65% (95% CI 8%–87%) for 4 doses. Among children ≥5 years of age, efficacy for 5 doses was 91% (95% CI 68%–98%) (Table 2).

Conclusions

Our results indicate that in Hyderabad, diphtheria mainly affected children 5–19 years of age, girls and women, and the Muslim population. Receiving a fourth and fifth doses of the vaccine was needed for protection against the disease. Coverage of primary vaccination was adequate in the city whereas, coverage for the boosters was low.

Low booster coverage, especially among Muslims, might have influenced herd immunity and thereby contributed to higher attack rates among this community. This factor was likely an important reason for persistence of diphtheria in Hyderabad. Similar phenomena were observed in countries where diphtheria reemerged after successful con-

Table 2. Number of doses of diphtheria vaccine received by diphtheria case-patients and matched controls, Hyderabad, India, 2006*

No. doses received	Case-patients			Controls			Odds ratio estimate	Vaccine efficacy estimate, % (95% CI)
	<5 y of age	≥5 y of age	Total	<5 y of age	≥5 y of age	Total		
0	6	20	26	2	11	13	Reference	Reference
1	0	7	7	1	2	3	1.4 (0.22–8.9)	0 (0–78)
2	2	6	8	0	2	2	2.1 (0.37–12)	0 (0–63)
3	6	34	40	8	26	34	0.51 (0.20–1.3)	49 (0–80)
4†	7	27	34	10	30	40	0.35 (0.13–92)	65 (8–87)
5‡	NA	8	8	NA	31	31	0.09 (0.02–0.33)	91§ (68–98)

*Conditional logistic regression taking into account vaccine doses, religion, family income, and parental literacy. CI, confidence interval; NA, not applicable.
†Booster at 18 mo of age.
‡Booster at 5 y of age.
§Vaccine efficacy among children ≥5 y of age; younger children could not have received that booster.

trol with vaccination (*2*,*7*). Several studies have reported vaccine efficacies ranging from 95% to 98% for 3 doses and from 90% to 99.9% for 5 doses (*8*,*9*). Two factors may explain the lower efficacy observed in our study. First, misclassification may have occurred when assessing vaccination status of children that mainly relied on a mother's recollection of the child's history. Second, program quality issues in vaccine supply or in cold-chain maintenance may have affected efficacy in Hyderabad. However, an evaluation of the universal immunization program conducted in 2006 in Hyderabad did not identify any gaps in cold-chain maintenance in the public health sector (Government of Andhra Pradesh, unpub. data). Thus, we concluded that misclassification with respect to assessment of vaccination status (using only the mother's recollection) probably explains the low vaccine efficacy that we observed.

Two factors could also explain the lower booster coverage among Muslims: 1) issues concerning the offer of vaccine by the health services or 2) issues concerning vaccine demand. However, primary vaccination coverage was identical among Muslims and non-Muslims in Hyderabad. This suggests that, initially, the demand for primary vaccination was identical in all communities, but that the health system was not able to retain the same demand for boosters in the Muslim community.

Our study had 2 main limitations. First, we only included patients admitted to Fever Hospital. Patients with milder symptoms who might not have sought treatment at the hospital were not considered. This situation may have led to an underestimation of attack rates but would not have led to different conclusions about the persistence of the disease. Second, a large proportion of children did not have vaccination cards, which may also have affected our vaccine efficacy estimates. We tried to address this factor by comparing vaccination status with the child's developmental milestones**.**

On the basis of our study results, we propose recommendations for control of diphtheria in Hyderabad. First, coverage for boosters must be improved, with special emphasis on the 4 administrative areas with high attack rates. Such efforts should be conducted among the Muslim community in particular. Second, mothers must be made more aware of the importance of booster doses. Again, these efforts should focus on the Muslim community. Third, because attack rates were high among adolescents, tetanus toxoid (administered to school children at 10–15 years of age) could be replaced with a combined tetanus-diphtheria vaccine. Fourth, coverage of boosters could be considered as performance indicators to improve the immunization program.

Acknowledgments

We are grateful to the Government of Andhra Pradesh, particularly I.V. Subba Rao and C. B. S. Venkata Ramana, for permission to conduct the study. We also thank P. Venkateswara Rao, R. Gopala Krishna Rao, B. Sai Babu, P. Manickam, R. Ramakrishnan, and K. Ramachandran for their help and support.

Dr Bitragunta is a medical officer in the Directorate of Health Services, Government of Andhra Pradesh. This study is based on Dr Bitragunta's dissertation work in the Master of Applied Epidemiology Programme (Field Epidemiology Training). Her research interests include diphtheria, tuberculosis, and maternal and child health.

References

1. World Health Organization. Diphtheria vaccine—WHO position paper. Wkly Epidemiol Rec. 2006;81:24–31.
2. World Health Organization. The immunological basis for immunization series. Module 2: diphtheria. Geneva: The Organization; 1993.
3. Chen RT, Broome CV, Weinstein RA, Weaver R, Tsai TF. Diphtheria in the United States, l971–l981. Am J Public Health. 1985;75:1393–7.
4. World Health Organization. Vaccine preventable diseases: monitoring system 2006 global summary. 2006 [cited 2008 May 23]. Available from http://www.who.int/vaccines-documents/GlobalSummary/GlobalSummary.pdf
5. Central Bureau of Health Intelligence. National health profile 2005. In: Health status indicators: Director General of Health Services, Government of India; Ministry of Health & Family Welfare Nirman Bhawan, New Delhi, 2006 [cited 2008 May 23]. Available from http://cbhidghs.nic.in/CBHI%20Book/chapter3.pdf
6. Orenstein WA, Bernier RH, Dondero TJ, Hinman AR, Marks JS, Bart KJ, et al. Field evaluation of vaccine efficacy. Bull World Health Organ. 1985;63:1055–68.
7. Galazka A. Implications of the diphtheria epidemic in the former Soviet Union for immunization programs. J Infect Dis. 2000;181(Suppl 1):S244–8. DOI: 10.1086/315570
8. Chen RT, Hardy IRB, Rhodes PH, Tyshchenko DK, Moiseeva AV, Marievsky VF. Ukraine, 1992: first assessment of diphtheria vaccine effectiveness during the recent resurgence of diphtheria in the former Soviet Union. J Infect Dis. 2000;181(suppl 1):S178–83. DOI: 10.1086/315561
9. Bisgard KM, Rhodes P, Hardy IRB, Litkina IL, Filatov NN, Monisov AA, et al. Diphtheria toxoid vaccine effectiveness: a case-control study in Russia. J Infect Dis. 2000;181(Suppl 1):S184–7. DOI: 10.1086/315562

Address for correspondence: Mohan D. Gupte, National Institute of Epidemiology, R-127, Tamilnadu Housing Board, Ayapakkam, Chennai-600 077, Tamilnadu, India; email: directorne@dataone.in

Anaplasma phagocytophilum Infection in Small Mammal Hosts of *Ixodes* Ticks, Western United States

Janet E. Foley,* Nathan C. Nieto,* Jennifer Adjemian,* Haydee Dabritz,† and Richard N. Brown‡

A total of 2,121 small mammals in California were assessed for *Anaplasma phagocytophilum* from 2006 through 2008. Odds ratios were >1 for 4 sciurids species and dusky-footed woodrats. High seroprevalence was observed in northern sites. Ten tick species were identified. Heavily infested rodent species included meadow voles, woodrats, deer mice, and redwood chipmunks.

Anaplasma phagocytophilum is a tick-transmitted pathogen that causes granulocytic anaplasmosis in humans, horses, and dogs (*1*–*3*). *A. phagocytophilum* is maintained in rodent-*Ixodes* spp. tick cycles, including the western blacklegged tick (*Indopacetus pacificus*) in the western United States (*4*). Transovarial transmission does not occur, and *I. pacificus* feeds only 1 time per stage, so infection must be acquired by a juvenile tick feeding on an infected mammal. Suggested reservoirs in the West include the dusky-footed woodrat (*Neotoma fuscipes)*, for which chronic infection has been observed, and the western gray squirrel (*Sciurus griseus*), which are frequently infected in nature (*5*,*6*). The northern coast range and Sierra Nevada foothills of California (*4*,*7*), where abundant rodents include deer mice (*Peromyscus* spp.), woodrats, and chipmunks (*Tamias* spp.), have moderate to high levels of granulocytic anaplasmosis. We sought to evaluate granulocytic anaplasmosis exposure and infection and describe the *Ixodes* spp. tick fauna in small mammals from central and northern coastal California.

The Study

Small mammals were caught in live traps (HB Sherman, Tallahassee, FL, USA, and Tomahawk Live Trap, Tomahawk, WI, USA) at 9 sites or collected as carcasses on roads (online Technical Appendix, available from www.cdc.gov/EID/content/14/7/1147-Techapp.pdf) from 2006 to 2008. Traps were set at locations of observed active rodent use or dens and baited with peanut butter and oats or corn, oats, and barley. Rodents were anesthetized with ketamine and xylazine delivered subcutaneously, examined for ectoparasites, and bled by retro-orbital abrasion or femoral venipuncture. The blood was anticoagulated with EDTA. Shrew (*Sorex* spp.) carcasses were retrieved when found in traps, kept cold, and then sampled in the laboratory. Live shrews were examined for ticks but released without further processing. All carcasses were identified to species, age, and sex; examined for ectoparasites; and then dissected for coagulated heart blood and spleen. Ectoparasites were preserved in 70% ethanol for identification. Data were included for animals from 3 previous studies (*5*,*8*,*9*).

Plasma anti–*A. phagocytophilum* immunoglobulin G (Ig) was assayed by an indirect immunofluorescent antibody assay (*3*), by using *A. phagocytophilum*–infected HL-60 cells as substrate and fluorescein isothiocyanate–labeled goat anti-rat heavy and light chain IgG (Kirkegaard and Perry, Gaithersburg, MD, USA). This assay does not distinguish exposure to *A. phagocytophilum* from *A. platys,* but the PCR was specific for *A. phagocytophilum.* PCR was performed for all flying (*Glaucomys sabrinus*), Douglas (*Tamiasciuris douglasii)*, and gray squirrels; all chipmunks from Santa Cruz and Marin Counties; a random subset of chipmunks from Humboldt Redwoods State Park and Hendy Woods State Park; and a random subset of individual mammals of other species. DNA was extracted from whole blood by using a kit (DNeasy Tissue kit, QIAGEN, Valencia, CA, USA), and real-time PCR was performed as described previously (*5*).

Data were analyzed with "R" (www.r-project.org), with a cutoff for statistical significance of $p = 0.05$. Differences in seroprevalence among small mammal species and between sexes were assessed by χ^2 test. Individual small mammals' risk for *A. phagocytophilum* exposure and infection were assessed as a function of sex, species, and location by calculating odds ratios (OR) and 95% confidence intervals (CI). Multivariate logistic regression was performed to evaluate seropositivity as a function of site, host species, and interactions to evaluate possible interaction and confounding between the variables.

A total of 2,121 small mammals, including 2,100 rodents, 20 shrews, and 1 lagomorph, were evaluated for exposure to and infection with *A. phagocytophilum* and infestation with *Ixodes* spp. ticks (Table 1). The overall seroprevalence was 15.2% (95% CI 13.6–16.9). Highest values and ORs >1 occurred in dusky-footed woodrats, tree squirrels, and some chipmunk species (Table 1; online Technical Appendix). The PCR prevalence among rodents

*University of California, Davis, California, USA; †California Department of Public Health, Richmond, California, USA; and ‡Humboldt State University, Arcata, California, USA

DOI: 10.3201/eid1407.071599

Table 1. Seroprevalence and PCR prevalence of *Anaplasma phagocytophilum* among small mammal species, northern and central coastal California*

Mammal species	*A. phagocytophilum* IFA			*A. phagocytophilum* msp2 PCR		
	Seropositive	Seroprevalence	95% CI	PCR positive	PCR prevalence	95% CI
Clethrionomys californicus	1	12.50	0.6–53.3	0	0	0–53.7
Glaucomys sabrinus	2	14.29	2.5–43.9	1	16.7	0.8–63.5
Mus musculus	0	0.00	0–25.3	0	0	0–34.4
Microtus californicus	2	5.88	1.0–21.1	0	0	0–17.8
Neotoma cinerea	0	0.00	0–94.5	0	0	0–94.5
N. fuscipes	167	50.15	44.7–55.6	8	4.3	2.0–8.6
N. macrotis	2	3.03	5.3–11.5	1	1.8	0.09–10.6
All *Neotoma*	169	42.25	37.4–47.3	9	3.7	1.8–7.1
Peromyscus boylii	3	8.82	2.3–24.8	1	4.0	0.2–22.3
P. californicus	2	0.67	0.1–2.7	0	0	0–3.8
P. maniculatus	18	3.46	2.1–5.5	0	0	0–6.6
P. truei	1	2.56	0.1–15.1	NT		
Peromyscus spp.	0	0.00	0–53.7	NT		
All *Peromyscus*	24	2.68	1.8–4.0	1	0.45	0.02–2.9
Rattus rattus	0	0.00	0–37	0	0	0–37.1
Reithrodontomys megalotis	0	0.00	0–17.2	1	6.3	0.3–32.3
Spermophilus beecheyi	0	0.00	0–4.2	0	0	0–20.0
S. lateralis	2	22.22	3.9–59.9	NT		
Sciurus carolinensis	11	57.89	34.0–78.9	3	18.8	5.0–46.3
S. griseus	34	70.83	55.7–82.6	6	15.8	6.6–31.9
S. niger	1	100.00	55.0–100.0	0	0	0–94.5
All *Sciurus*	46	47.83	33.1–62.9	9	16.4	8.2–29.3
Sorex spp.	0	0.00	0–37.0	0	0	0–94.5
Sylvilagus bachmani	0	0.00	0–94.5	NT		
Tamias amoenus	6	6.82	2.8–14.8	NT		
T. merriami	0	0.00	0–48.3	0	0	0–40.2
T. minimus	0	0.00	0–4.9	NT		
T. senex	5	4.81	1.8–11.4	NT		
T. speciosus	4	33.33	11.3–64.6	NT		
T. sonomae	1	14.29	0.7–58.0	2	50.0	15.0–85.0
T. ochrogenys	30	27.52	19.6–37.0	2	6.9	1.2–24.2
Tamias spp.	2	8.33	1.5–28.5	NT		
All *Tamias*	48	13.45	10.2–17.5	4	34.0	3.2–24.1
Tamiasciurus douglasii	6	40.00	17.5–67.1	0	0	0–60.4
Total	300	15.24	13.7–16.9	33	3.8	2.9–5.3

*IFA, immunofluorescence assay; CI, confidence interval; NT, not tested.

tested was 3.8% (N = 652, 95% CI 2.9–5.3); highest values were reported in tree squirrels and some chipmunk species (Table 1). Although deer mice have been reported to be exposed to *A. phagocytophilum* (*10*,*11*), we found little evidence of this in our study. Woodrats at northern sites tended to be infected, while sciurids (excluding ground squirrels) showed high rates of exposure at multiple sites, consistent with previous reports (*5*). A total of 60% of eastern gray squirrels from Connecticut were seropositive with reservoir competence documented by producing PCR-positive ticks after feeding on infected squirrels (*12*). A PCR-positive eastern chipmunk (*Tamias striatus*) was reported from Minnesota (*13*).

Location was an important determinant of exposure to infection, with high seroprevalence in the Hoopa Valley Indian Reservation and Hendy Woods State Park (Table 2). ORs significantly <1 were observed for Samuel P. Taylor State Park and the Morro Bay area, and 5 sites in the far northern coast range and Quincy in the Sierra Nevada had ORs >1 (online Technical Appendix). Statistical analysis failed to document a significant interaction between site and host species, but confounding was apparent, with overrepresentation of gray squirrels and woodrats in some high prevalence sites (online Technical Appendix). PCR prevalence was high at Sutter Buttes State Park and Siskiyou County (both with low sample size) and Big Basin State Park and Hendy Woods State Park, each ≈12% (Table 2). Results are consistent with prior reports for horses and dogs (*4*). Previous spatial analysis documented increased *A. phagocytophilum* risk in redwood, montane hardwood, and blue oak/foothill pine habitats (*14*). In our dataset, obvious habitat differences would not account for differences in disease exposure, given the presence of live oak, tanoak, redwood, and Douglas fir at many sites. Further ecologic studies to identify differing ecologic factors among these sites would be useful.

Tick species observed in our study sites include possible enzootic vectors and several human-biting species, including *I. pacificus* and *I. angustus* (online Technical Appendix). Host species from which relatively large col-

Table 2. Regional seroprevelance and PCR prevalence rates for exposure to *Anaplasma phagocytophilum* in small mammals in various sites, northern and central California*

Site	*A. phagocytophilum* IFA			*A. phagocytophilum* msp2 PCR		
	Seropositive	Seroprevalence	95% CI	PCR positive	PCR prevalence	95% CI
Big Basin State Park	16	6.30	3.76–10.22	5	12.20	4.58–27.00
Humboldt Redwoods State Park	24	16.90	11.33–24.31	2	6.06	1.06–21.62
Hoopa Valley Indian Reservation	173	36.19	31.91–40.70	6	4.14	1.69–9.18
Hendy Woods State Park	43	22.51	16.93–29.22	5	12.19	4.58–27.00
King Range National Conservation Area	1	3.45	0.18–19.63	0	0.00	0.00–80.21
Mendocino County (roadside only)	0	0.00	0.00–94.53	0	0.00	0.00–94.54
Morro Bay regional communities	5	1.23	0.45–3.01	2	0.67	0.12–2.65
Placerville City region (roadside only)	1	1.00	5.46–1.00	1	1.00	5.46–1.00
Quincy City region (roadside only)	2	50.00	15.00–84.99	0	0.00	0.00–60.42
Sutter Buttes State Park	3	7.50	1.96–21.48	1	50.00	9.45–90.55
Sagehen Research Station	17	7.69	4.68–12.24	0	0.00	0.00–60.42
Siskiyou County (roadside only)	3	1.00	30.99–1.00	1	33.33	1.76–87.47
Sonoma	1	1.00	5.46–1.00	0	0.00	0.00–94.54
Samuel P. Taylor State Park	3	1.75	0.42–5.45	2	4.26	0.74–15.73
Trinity County (roadside only)	2	40.00	7.26–82.96	0	0.00	0.00–53.71
Sacramento River Valley (roadside only)	3	1.00	30.99–1.00	0	0.00	0.00–69.00
Willow Creek Town (roadside only)	3	0.30	8.09–64.63	0	0.00	0.00–60.42
Yolo County	1	6.67	0.35–33.97	0	0.00	0.00–25.35

*IFA, immunofluorescence assay; CI, confidence interval.

lections were obtained included meadow voles, woodrats, deer mice, tree squirrels, and redwood chipmunks (*T. ochrogenys)*. Tick diversity was highest on redwood chipmunks and in more northerly sites (online Technical Appendix). *I. angustus,* primarily a nidicolous tick of rodents but occasionally bites humans and is a competent vector for *Borrelia burgdorferi* sensu stricto (*15*), occurred on most rodent species. *I. spinipalpis,* which occurred on woodrats, deer mice, squirrels, and chipmunks, functions as a primary vector for *B. bissettii* in a woodrat enzootic cycle (*16*), and *Neotoma mexicana* and *I. spinipalpis* have an enzootic cycle in Colorado for *A. phagocytophilum*.

Conclusions

We show that a strong distinction can be made in possible reservoir capacity among rodent species, with many, such as deer mice and voles, only contributing to the ecology of granulocytic anaplasmosis through their support of ticks but not *A. phagocytophilum* infection. Others, including tree squirrels and woodrats, are frequently infected, in addition to supporting ticks. Considerable similarities exist between the ecology of *A. phagocytophilum* and *B. burgdorferi* in the West, although the large diversity of genospecies that exists for *B. burgdorferi* has not been reported for *A. phagocytophilum.* These data provide a starting point for future work to clarify the reservoir competence of small mammals for *A. phagocytophilum* and to determine how ecologic interactions among small mammals, other vertebrate hosts, multiple possible vectors, and both *B. burgdorferi* and *A. phagocytophilum* could affect the enzootic persistence of these pathogens and risk to humans and animals.

Acknowledgments

We thank Niki Drazenovich, Elizabeth Holmes, Bernadette Clueit, Michael Adjemian, Jamie Bettaso, Greta Wengert, and Edwin Saada for field and laboratory assistance; and Patrick Foley for suggestions for analysis. Personnel at each of the study sites (Hoopa Valley Indian Tribe, UC Reserve System, California State Parks, Bureau of Land Management, and private land owners) provided invaluable access and logistic support.

Financial support was provided by the UC Davis Center for Vectorborne Diseases and the Committee on Research.

Dr Foley is a disease ecologist at the University of California, Davis, Department of Medicine and Epidemiology. She is also a veterinarian. Her major research focus is the ecology of tick-borne diseases.

References

1. Greig B, Asanovich KM, Armstrong PJ, Dumler JS. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. J Clin Microbiol. 1996;34:44–8.
2. Madigan JE. Equine ehrlichiosis. Vet Clin North Am Equine Pract. 1993;9:423–8.
3. Dumler JS, Asanovich KM, Bakken JS, Richter P, Kimsey R, Madigan JE. Serologic cross-reactions among *Ehrlichia equi, Ehrlichia phagocytophila,* and human granulocytic ehrlichia. J Clin Microbiol. 1995;33:1098–103.

4. Foley JE, Foley P, Brown RN, Lane RS, Dumler JS, Madigan JE. Ecology of granulocytic ehrlichiosis and Lyme disease in the western United States.. J Vector Ecol. 2004;29:41–50.
5. Nieto NC, Foley J. Evaluation of squirrels as ecologically significant hosts for *Anaplasma phagocytophilum* in California. J Med Entomol. In press.
6. Foley JE, Kramer VL, Weber D. Experimental ehrlichiosis in dusky footed woodrats (*Neotoma fuscipes*). J Wildl Dis. 2002;38:194–8.
7. California Department of Health Service. Vector-borne diseases in California. 2004 annual report. Sacramento (CA): The Department; 2006.
8. Foley J, Clueit S, Brown RN. Differential exposure to *Anaplasma phagocytophilum* in rodent species in northern California. Vector Borne Zoonotic Dis. 2008;8:49–55. DOI: 10.1089/vbz.2007.0175
9. Nieto NC, Foley P, Calder L, Dabritz H, Adjemian J, Conrad PA, et al. Ectoparasite diversity and exposure to vector-borne disease agents in wild rodents in central coastal California. J Med Entomol. 2007;44:328–35. DOI: 10.1603/0022-2585(2007)44[328:EDAETV]2.0.CO;2
10. Zeidner NS, Burkot TR, Massung R, Nicholson WL, Dolan MC, Rutherford JS, et al. Transmission of the agent of human granulocytic ehrlichiosis by *Ixodes spinipalpis* ticks: evidence of an enzootic cycle of dual infection with *Borrelia burgdorferi* in Northern Colorado. J Infect Dis. 2000;182:616–9. DOI: 10.1086/315715
11. Nicholson WL, Muir S, Sumner JW, Childs JE. Serologic evidence of infection with *Ehrlichia* spp. in wild rodents (Muridae: Sigmodontinae) in the United States. J Clin Microbiol. 1998;36:695–700.
12. Levin ML, Nicholson WL, Massung RF, Sumner JW, Fish D. Comparison of the reservoir competence of medium-sized mammals and *Peromyscus leucopus* for *Anaplasma phagocytophilum* in Connecticut. Vector Borne Zoonotic Dis. 2002;2:125–36. DOI: 10.1089/15303660260613693
13. Walls JJ, Greig B, Neitzel D, Dumler J. Natural infection of small mammal species in Minnesota with the agent of human granulocytic ehrlichiosis. J Clin Microbiol. 1997;35:853–5.
14. Foley JE, Queen E, Sacks B, Foley P. GIS-facilitated spatial epidemiology of tick-borne diseases in coyotes (*Canis latrans*) in northern and coastal California. Comp Immunol Microbiol Infect Dis. 2005;28:197–212. DOI: 10.1016/j.cimid.2005.01.006
15. Peavey CA, Lane RS, Damrow T. Vector competence of *Ixodes angustus* (Acari: Ixodidae) for *Borrelia burgdorferi* sensu stricto. Exp Appl Acarol. 2000;24:77–84. DOI: 10.1023/A:1006331311070
16. Brown RN, Peot MA, Lane RS. Sylvatic maintenance of *Borrelia burgdorferi* (Spirochaetales) in Northern California: untangling the web of transmission. J Med Entomol. 2006;43:743–51. DOI: 10.1603/0022-2585(2006)43[743:SMOBBS]2.0.CO;2

Address for correspondence: Janet E. Foley, Department of Medicine and Epidemiology, University of California, Davis, California 95616, USA; email: jefoley@ucdavis.edu

Risk for Avian Influenza Virus Exposure at Human–Wildlife Interface

Jennifer Siembieda,* Christine K. Johnson,* Walter Boyce,* Christian Sandrock,† and Carol Cardona*

To assess risk for human exposure to avian influenza viruses (AIV), we sampled California wild birds and marine mammals during October 2005–August 2007and estimated human–wildlife contact. Waterfowl hunters were 8 times more likely to have contact with AIV-infected wildlife than were persons with casual or occupational exposures ($p<0.0001$).

The emergence of highly pathogenic avian influenza virus (AIV) (H5N1) in domestic poultry in Asia with spillover infections in humans has raised concerns about the potential for a human pandemic (*1*). Although subtype H5N1 is the most well-known infecting strain, evidence of direct bird-to-human transmission has been documented for several other AIV subtypes (*2*).

Little is known about the types of exposure that result in human infections, especially with AIV being transmitted from wild birds and animals because only a few cases of transmission to humans have been documented (*3*–*5*). Overall, the types of exposures associated with the transmission of AIV to humans have been ingestion, inhalation of aerosolized virus, or direct contact through mucous membranes (2,*4*). The probability of infection with AIV varies with the activity and depends on the contact type (duration and route) and dose. Contacts for the general public are likely short and indirect, often occurring through outdoor activities, such as hiking, picnicking, or feeding birds. Contact for waterfowl hunters is especially intense and direct during bird-cleaning activities. Biologists and workers at wildlife hospitals have frequent and direct contact with wild birds and mammals. Biologists trap apparently healthy free-ranging animals and perform field necropsies, and rehabilitation workers handle sick and injured wild animals. In this study, we tested wild birds and marine mammals for AIV to determine the exposure risks associated with specific casual, recreational, and occupational activities that result in contact with wildlife.

The Study

Human risk categories were created based on a typical contact type with wildlife: 1) casual (the general public), 2) recreational (waterfowl hunters), and 3) occupational (wildlife biologists, wildlife hospital workers, and veterinarians). Frequency of contact with AIV was estimated for each risk group by evaluating the prevalence of AIV among animals sampled opportunistically in each category. Surveillance for AIV was conducted from October 2005 through August 2007.

For casual contact, wild bird species (mostly periurban passerines such as sparrows, finches, and crows) were sampled to reflect typical daily exposures for the public (Figure). For recreational contact, birds were assessed by sampling hunter-killed waterfowl (mostly mallards, northern shovelers, gadwalls, green-winged teals, northern pintails, and American widgeons) at check stations in the Sacramento National Wildlife Refuge. For occupational contact, wild birds (seabirds, wading birds, waterfowl, raptors, and passerines) and marine mammals (seals and sea lions) admitted to 3 northern California wildlife hospi-

Figure. Map of California displaying sample collection sites for avian influenza testing, fall 2005–summer 2007. The casual risk category is represented by a square, recreational risk category by a star, and occupational risk category by a circle. Counties are abbreviated as follows: CC, Contra Costa; GLE, Glenn; KER, Kern; LA, Los Angeles; MRN, Marin; ORA, Orange; RIV, Riverside; SAC, Sacramento; SOL, Solano; YOL, Yolo.

*University of California, Davis, School of Veterinary Medicine, Davis, California, USA; and †University of California, Davis, School of Medicine, Sacramento, California, USA

DOI: 10.3201/eid1407.080066

tals were sampled. Cloacal samples were taken from birds and nasal and rectal samples from marine mammals with rayon-tipped swabs (MicroPur; PurFybr, Inc., Munster, IN, USA). Birds in recovery also had oropharyngeal samples taken. Swab samples were placed in viral transport media, transported within 24 hours from the site of collection to the University of California, Davis, in a cooler with ice packs and then transferred to a –70°C freezer for storage. A total of 9,157 samples were tested for AIV. Of these, 2,346 were screened by virus isolation in embryonating chicken eggs (*6*,*7*), and 6,811 were screened by real-time reverse transcription–PCR (RT-PCR) (*7*). All positive samples were tested for Eurasian H5 viruses (*8*).

The prevalence of AIV in each group was low (range 0.1%–0.9%) (Table), and no samples were positive for Eurasian H5. We found that risk of contact with AIV-infected wildlife was 8 times higher for the recreational group compared to either the occupational or the casual group ($p<0.0001$; EpiInfo, Centers for Disease Control and Prevention, Atlanta, GA, USA).

Conclusions

We did not detect AIV (H5N1) in California during October 2005–August 2007 nor did other surveillance efforts in the United States (*9*). We did detect other AIVs, although at a low prevalence (<1%). The prevalence of AIV in California wildlife was substantially lower than the prevalence reported in Alaskan wildlife in the same flyway (*10*). AIV prevalence may decrease with latitude (*11*), or this opportunistic sample design may have resulted in testing of species with a natural low prevalence. Although overall prevalence was low, it was highest in the recreational category and, coupled with the directness and intensity of the contacts especially during bird cleaning, this group would be expected to have the highest risk for infection. However, emergence or introduction of a virus that causes disease in wild birds or animals would likely result in a disproportional shift in prevalence of infection in wildlife brought to rehabilitation hospitals, thus making occupational contact more risky. As a recent example, 1 stork and 2 buzzards that were infected with AIV (H5N1) were brought to a wildlife hospital in Poland, which potentially exposed staff (*12*).

Novel transmission pathways are possible in places like wildlife hospitals because wild species that do not meet in nature are brought into close and extended contact with each other and humans. For example, marine mammals are susceptible to infection with AIV (*4*) and human influenza viruses (*13*) and have been documented as intermediate hosts (*4*). Other species may also be intermediate hosts for AIV, although they have not been identified. Those working in wildlife occupations should be encouraged to wear personal protective equipment when handling wildlife because of the types of contacts they can have and the potential for viruses to emerge in this setting. Similarly, personal protection should be recommended for waterfowl hunters because of the relatively higher prevalence of AIV in the birds with which they have contact.

We assessed the risk for human exposure to AIV by opportunistically sampling wildlife at the human–wild animal interface. A better measure of human risk would be to directly assess human exposure by testing for antibodies to all AIV subtypes that could occur in nature. Although it is not practical to simultaneously test for 144 virus subtypes, 2 serologic studies of persons exposed to wildlife showed antibodies to a limited number of AIVs (*3*,*14*). Since these exposures did not cause discernable illness, diagnosis based on clinical signs would likely underestimate infection.

Although our methods enabled us to compare exposure risk among different groups, the testing methods we used likely did not estimate the true AIV prevalence in wildlife. The real-time RT-PCR used in this study and in national surveillance efforts (*7*) has not been validated in wildlife (*10*), nor has virus isolation in embryonating chicken eggs, and it may be that neither method is perfect in detecting AIV in species that are only distantly related to chickens (*15*). Improved diagnostic methods are needed to assess AIV infections in wildlife species, and close monitoring of persons with the highest level of exposure to AIV is a necessary component of an early warning system to detect transmission from animals to humans.

Acknowledgments

We gratefully acknowledge the expert technical assistance provided by Nichole Anchell, Nguyet Dao, Phuong Dao, Grace Lee, and Jerome Anunciacion. We also appreciate those who provided assistance with sample collection: Michelle Bellizzi, Marie Travers, and Megan Prelinger; January Bill and Shannon Riggs (Oiled Wildlife Care Network); Susan Heckly, Marcia Metzler, Sandy Fender, Pam Nave, Jean Yim, Devin Dombrowski, Rachel Avilla, and Jarl Rasmussen (Lindsay Wildlife Museum); Frances Gulland, Felicia Nutter, Amber Makie, Tanaya Norris, Tracey Goldstein, Michelle Blascow, and Liz Wheeler (Marine Mammal

Table. Prevalence of avian influenza viruses in California wild birds and marine mammals, October 2005–August 2007, categorized by exposure risk category

Exposure risk group	No. positive (%)	No. tested	Species (no. positive)
Casual	8 (0.2)	4,757	Finch (3), sparrow (2), cowbird (1), quail (2)
Recreational	20 (0.9)	2,346	Duck (19), goose (1)
Occupational	2 (0.1)	2,054	Seabird (1), egret (1)
Total	30 (0.3)	9,157	

Center); William Reisen (UC Davis Center for Vectorborne Diseases); Robert Cummings (Orange County Mosquito and Vector Control District); Stan Wright (Sacramento/Yolo Mosquito and Vector Control District); and Terra Kelly and Julie Nelson (UC Davis Wildlife Health Center). In addition, we thank the Sacramento National Wildlife Refuge and California Department of Fish and Game for assistance with logistics and obtaining samples at the refuge.

Support for this project was provided by the Avian Influenza Coordinated Agricultural Project (US Department of Agriculture/Cooperative State Research, Education, and Extension Service grant 2005-35605-15388), "The Prevention and Control of Avian Influenza in the United States," the National Institute of Allergy and Infectious Diseases, National Institutes of Health, the Department of Health and Human Services (contract no. HHSN266200700009C), the Resource Assessment Program of the California Department of Fish and Game, and the Edward E. Hills Foundation.

Dr Siembieda is a veterinarian and a graduate researcher in epidemiology at the Wildlife Health Center of the University of California in Davis. Her primary research interests include the epidemiology and ecology of zoonotic pathogens at the human–wildlife interface, namely avian influenza and enteric, bacterial, and protozoal pathogens.

References

1. Capua I, Marangon S. Control of avian influenza in poultry. Emerg Infect Dis. 2006;12:1319–24.
2. Sandrock C, Kelly T. Clinical review: update of avian influenza A infections in humans. Crit Care. 2007;11:209. DOI: 10.1186/cc5675
3. Gill JS, Webby R, Gilchrist MJ, Gray GC. Avian influenza among waterfowl hunters and wildlife professionals. Emerg Infect Dis. 2006;12:1284–6.
4. Webster RG, Geraci J, Petursson G, Skirnisson K. Conjunctivitis in human beings caused by influenza A virus of seals. N Engl J Med. 1981;304:911.
5. World Health Organization. Outbreak news. Avian influenza, Azerbaijan. Wkly Epidemiol Rec. 2006;81:183–8.
6. Swayne DE, Senne DA, Beard CW. Avian influenza. In: Swayne DE, Glisson FR, Jackwood MW, Pearson JE, Reed WM, Reed WM, editors. Laboratory manual for the isolation and identification of avian pathogens. Kennett Square (PA): American Association of Avian Pathologists; 1998. p. 150–5.
7. Spackman E, Senne DA, Bulaga LL, Myers TJ, Perdue ML, Garber LP, et al. Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis. 2003;47(Suppl):1079–82. DOI: 10.1637/6091
8. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 2002;40:3256–60. DOI: 10.1128/JCM.40.9.3256-3260.2002
9. US Geological Survey National Wildlife Health Center. Highly pathogenic avian influenza early detection data system [cited 2007 Nov 17]. Available from http://wildlifedisease.nbii.gov/ai/index.jsp
10. Runstadler JA, Happ GM, Slemons RD, Sheng ZM, Gundlach N, Petrula M, et al. Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. Arch Virol. 2007;152:1901–10. DOI: 10.1007/s00705-007-0994-1
11. Stallknecht DE, Shane SM. Host range of avian influenza virus in free-living birds. Vet Res Commun. 1988;12:125–41. DOI: 10.1007/BF00362792
12. ProMED. H5N1 bird flu found at 5th site in Poland. 2007 [cited 2008 Jan 3]. Available from http://www.promedmail.org/pls/otn/f?p=2400:1202:1477266451936125:NO:F2400_P1202_CHECK_DISPLAY,F2400_P1202_PUB_MAIL_ID:X,40433
13. Ohishi K, Ninomiya A, Kida H, Park CH, Maruyama T, Arai T, et al. Serological evidence of transmission of human influenza A and B viruses to Caspian seals (*Phoca caspica*). Microbiol Immunol. 2002;46:639–44.
14. Myers KP, Setterquist SF, Capuano AW, Gray GC. Infection due to 3 avian influenza subtypes in United States veterinarians. Clin Infect Dis. 2007;45:4–9. DOI: 10.1086/518579
15. Capua I, Mutinelli F, Hablovarid MH. Avian embryo susceptibility to Italian H7N1 avian influenza viruses belonging to different genetic lineages. Arch Virol. 2002;147:1611–21. DOI: 10.1007/s00705-002-0837-z

Address for correspondence: Carol Cardona, Extension, Surge III, Rm 1383, School of Veterinary Medicine, University of California, Davis, CA 95616, USA; email: cjcardona@ucdavis.edu

Optimizing Use of Multistream Influenza Sentinel Surveillance Data

Eric H. Y. Lau,* Benjamin J. Cowling,* Lai-Ming Ho,* and Gabriel M. Leung*

We applied time-series methods to multivariate sentinel surveillance data recorded in Hong Kong during 1998–2007. Our study demonstrates that simultaneous monitoring of multiple streams of influenza surveillance data can improve the accuracy and timeliness of alerts compared with monitoring of aggregate data or of any single stream alone.

The use of separate data streams based on sentinel surveillance has long been an accepted approach to monitor community incidence and to enable timely detection of infectious disease outbreaks (*1*,*2*). Recently, more attention has been given to the combined analysis of multivariate sentinel data (*3*–*5*).

In this study we explored the possibility of improving the ability to more quickly detect peak periods of influenza activity in Hong Kong through simultaneous monitoring of multiple streams of sentinel surveillance data. Our findings have general implications in the choice of surveillance algorithms where multistream data are available.

The Study

The local Department of Health publishes weekly reports (*6*) from a network of 50 private-sector sentinel general practitioners (GP) and 62 public-sector sentinel general outpatient clinics (GOPC) on the proportion of patients seeking treatment for influenza-like illness (ILI), defined as fever plus cough or sore throat (*7*). In this study, we used the GP and GOPC sentinel surveillance data in 9 annual influenza seasons from 1998–1999 to 2006–2007, stratified by 4 geographic regions in Hong Kong—Hong Kong Island, Kowloon, New Territories East, and New Territories West—resulting in 8 separate data streams (Figure).

Each month a median of 1,555 specimens (interquartile range 1,140–2,740), primarily from hospitals, were sent to the Government Virus Unit of the Department of Health (*7*). We calculated the highest proportion of positive influenza isolations each season, and used these laboratory data to define the onset of each peak activity period when the proportion of positive influenza A or B isolates exceeded 30% of the maximum seasonal level (*7*).

Dynamic linear models (*8*) were used to generate alerts (online Technical Appendix, available from www.cdc.gov/EID/content/13/7/1154-Techapp.pdf). We determined that an aberration had occurred when the current observation fell outside a forecast interval generated by the model. For methods based on monitoring of single data streams only, an aberration triggers an alert. For simultaneous monitoring of all 8 data streams, we monitored separate aberrations as above and generated alerts based on the first occurrence of any aberration (M1), 2 simultaneous aberrations (M2), the first occurrence of 3 simultaneous aberrations (M3), any 2 aberrations within a 2-week period (M4), and any 3 aberrations within a 2-week period (M5). In the multistream analyses, we compared alerts produced by univariate models, which effectively assumed independence between the data streams, and multivariate models, which allowed for correlation between the data streams (online Technical Appendix).

Alerts were compared in terms of their sensitivity, specificity, and timeliness in detecting the onset of peak levels of influenza activity (*9*). We combined these metrics and estimated the area under the weighted receiver operating characteristic curve (AUWROC) as an overall measure of performance (*10*). The Table shows the highest AUWROC, for each method, from a predefined selection of parameter combinations and the sensitivity and timeliness at a fixed specificity of 95%. On the basis of aggregated data, we determined that alerts generated from the GOPC network achieved a higher AUWROC and better timeliness than those from the GP network. However, the best AUWROC from each of the data streams was produced by the GP New Territories East data, which outperformed the aggregate GP data. Conversely, for GOPC data, the performance of aggregate data was superior to that of any single data stream.

The Table also shows simultaneous monitoring results for all 8 geographic data streams from both GPs and GOPCs. For the univariate (independent) models for each data stream, methods based on simultaneous alerts perform well. The optimal methods were M2 and M3 with AUWROC of 0.89 and 0.90 and timeliness of 1.22 and 1.47 weeks, respectively, for a fixed specificity of 0.95. In general, univariate models performed better than multivariate models. Empirical correlation derived from one of the fitted multivariate models is shown in the online Technical Appendix; correlation structures under other models were similar (data not shown).

Results were insensitive to the choice of parameters (online Technical Appendix). The results also held when we varied the definition of the start of peak influenza activity between 10% and 50% of peak seasonal levels (online Technical Appendix).

*University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China

DOI: 10.3201/eid1407.080060

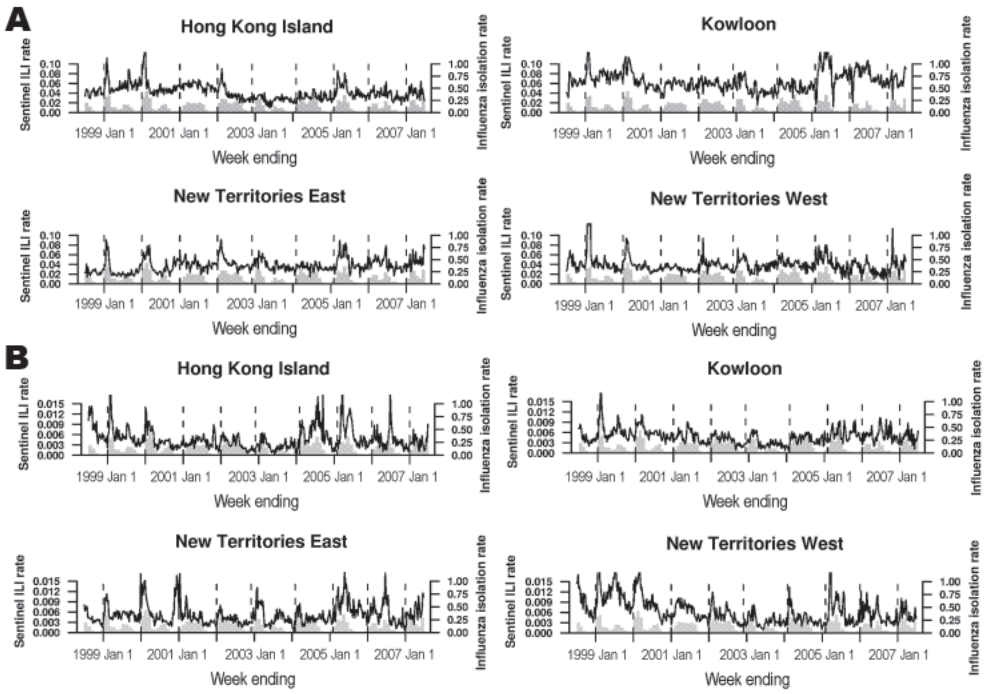


Figure. Nine annual cycles (unbroken lines) of general practitioner (A) and general outpatient clinic (B) geographic sentinel surveillance data from Hong Kong Island, Kowloon, New Territories East, and New Territories West, 1998–2007. The monthly proportions of laboratory samples testing positive for influenza isolates are overlaid as gray bars, and the beginning of each annual period of peak activity (inferred from the laboratory data) is marked with a vertical dotted line. ILI, influenza-like illness.

Conclusions

A primary objective of sentinel surveillance is to provide sensitive, specific, and timely alerts at the beginning of increased disease activity (*11*). We evaluated the performance of multistream sentinel surveillance of ILI in detecting the onset of peak influenza activity.

Splitting sentinel data into separate geographic-based streams and monitoring all 8 streams for 2 or 3 simultaneous aberrations provided substantial improvements in AUWROC and also in timeliness for a fixed specificity when compared with monitoring aggregated data or any single data stream. We also used multivariate models with various alternative correlation structures between data streams, but use of these more complex models did not appear to improve performance (Table), possibly because correlation between streams vary year to year; the multivariate model is based on constant correlations (online Technical Appendix). It is possible that other complex multivariate models may allow even greater improvement in performance; however, simultaneous monitoring of data streams may be more practical because univariate models may be applied in a spreadsheet (*7*).

Although the relative performance of GP and GOPC sentinels may not be directly generalizable to other settings with differences in infectious disease dynamics and healthcare systems, the implications for data collection are nevertheless relevant. Inclusion of data streams should be based on their value to the overall surveillance system, rather than independent performance. For example, simultaneous monitoring of data streams where some have lower specificity and others have higher specificity could still improve overall timeliness.

Specifically regarding Hong Kong, it is unclear why alerts from the private GP network have better timeliness than those from the public GOPC network. Although we note that both networks have different catchment populations, the GOPC network typically serves elderly and lower income groups (*12*), whereas influenza would be more likely to affect children at the start of the influenza season (*13*). Differences between geographic regions could be real, when disease progresses from 1 region to another (*14*); however, this circumstance is unlikely in Hong Kong, an area of only 1,000 km^2, where a high degree of mixing occurs among a population of 7 million persons. Geographic heterogeneity could also be explained by differential socioeconomics and demographics between different regions, associated differences in access to healthcare and health-seeking behavior issues, or small area variations in reporting behavior among the sentinel practices.

A potential caveat of our analysis is the small number of annual cycles of sentinel data available for study. However, until recently, few subtropical or tropical regions had begun influenza sentinel surveillance. Another limitation is the absence of a generally agreed-upon standard in defining a peak influenza season. In our analysis, the start of peak activity was defined as laboratory isolation rates exceeding 30% of the annual level; however, we found that our results were not sensitive to other reasonable thresholds. In addition, we compared methods with only a few chosen parameter combinations; sensitivity analyses showed that

Table. Performance of alerts generated by individual monitoring of aggregate data and separate data streams, and simultaneous monitoring of multiple data streams by using univariate and multivariate time series models, Hong Kong, 1998–2007*

	Univariate models			Multivariate models†		
Data	AUWROC	Sensitivity‡	Timeliness, wk‡	AUWROC	Sensitivity‡	Timeliness, wk‡
Aggregated data						
GP	0.78	1.00	2.41	–	–	–
GOPC	0.86	1.00	1.50	–	–	–
Single stream						
GP						
HK	0.75	1.00	2.36	0.73	0.87	2.64
KL	0.66	1.00	2.71	0.62	0.88	3.06
NTE	0.89	1.00	2.00	0.76	0.90	2.04
NTW	0.80	1.00	2.07	0.80	0.91	2.24
GOPC						
HK	0.79	1.00	2.21	0.71	0.89	2.42
KL	0.78	1.00	2.46	0.62	0.96	3.15
NTE	0.79	0.95	2.22	0.79	0.96	2.26
NTW	0.73	1.00	2.55	0.72	1.00	2.52
Multiple streams						
M1: First aberration	0.84	1.00	1.57	0.86	1.00	1.66
M2: 2 simultaneous aberrations	0.89	1.00	1.22	0.82	1.00	1.77
M3: 3 simultaneous aberrations	0.90	1.00	1.47	0.80	1.00	1.70
M4: Any 2 aberrations in 2 wk	0.81	1.00	2.63	0.72	1.00	2.43
M5: Any 2 aberrations in 2 wk	0.83	1.00	2.44	0.77	1.00	2.11

*AUWROC, area under the weighted receiver operating characteristic curve; GP, general practitioner; GOPC, general outpatient clinic; HK, Hong Kong Island; KL, Kowloon; NTE, New Territories East; NTW, New Territories West.
†See online Technical Appendix (available from www.cdc.gov/EID/content/14/7/1154-Techapp.pdf) for more detailed description of the multivariate model.
‡At a fixed specificity of 0.95.

the results were not sensitive to the smoothing parameter or the specification of correlations between streams. Finally, alerts generated by other more complicated combinations of aberrations might provide further enhancements. However, the value of simultaneously monitoring separate data streams (*15*) has already been demonstrated by the simple combinations chosen here.

Acknowledgments

We gratefully acknowledge the sentinel practitioners, who through their own goodwill, have been providing weekly data to the Hong Kong Department of Health for infectious disease surveillance. We also thank Irene Wong for technical assistance.

This research was in part funded by the Research Fund for the Control of Infectious Diseases of the Food and Health Bureau of the Hong Kong SAR Government (grant no. 04050102) and by the Area of Excellence Scheme of the University Grants Committee (grant no. AoE/M–12/06).

Dr Lau is a postdoctoral fellow in the School of Public Health at the University of Hong Kong. He is interested in the methodology and application of statistical and mathematical models to infectious diseases.

References

1. Besculides M, Heffernan R, Mostashari F, Weiss D. Evaluation of school absenteeism data for early outbreak detection, New York City. BMC Public Health. 2005;5:105. DOI: 10.1186/1471-2458-5-105
2. Goldenberg A, Shmueli G, Caruana RA, Fienberg SE. Early statistical detection of anthrax outbreaks by tracking over-the-counter medication sales. Proc Natl Acad Sci U S A. 2002;99:5237–40. DOI: 10.1073/pnas.042117499
3. Mohtashemi M, Kleinman K, Yih WK. Multi-syndrome analysis of time series using PCA: a new concept for outbreak investigation. Stat Med. 2007;26:5203–24. DOI: 10.1002/sim.2872
4. Reis BY, Kohane IS, Mandl KD. An epidemiological network model for disease outbreak detection. PLoS Med. 2007;4:e210. DOI: 10.1371/journal.pmed.0040210
5. Fricker RD. Directionally sensitive multivariate statistical process control procedures with application to syndromic surveillance. Advances in Disease Surveillance. 2007;3:1–17. Available from http://www.isdsjournal.org/article/view/3%3B1/831
6. Hutwagner LC, Maloney EK, Bean NH, Slutsker L, Martin SM. Using laboratory-based surveillance data for prevention: an algorithm for detecting *Salmonella* outbreaks. Emerg Infect Dis. 1997;3:395–400.
7. Cowling BJ, Wong IO, Ho LM, Riley S, Leung GM. Methods for monitoring influenza surveillance data. Int J Epidemiol. 2006;35:1314–21. DOI: 10.1093/ije/dyl162
8. West M, Harrison J. Bayesian forecasting and dynamic models. 2nd ed. New York: Springer; 1997.
9. Hutwagner L, Browne T, Seeman GM, Fleischauer AT. Comparing aberration detection methods with simulated data. Emerg Infect Dis. 2005;11:314–6.
10. Kleinman KP, Abrams AM. Assessing surveillance using sensitivity, specificity and timeliness. Stat Methods Med Res. 2006;15:445–64.
11. Lawson AB, Kleinman K. Spatial and syndromic surveillance for public health. West Sussex (UK): Wiley; 2005.
12. Leung GM, Wong IO, Chan WS, Choi S, Lo SV. The ecology of health care in Hong Kong. Soc Sci Med. 2005;61:577–90. DOI: 10.1016/j.socscimed.2004.12.029
13. Glezen WP, Couch RB. Interpandemic influenza in the Houston area, 1974–76. N Engl J Med. 1978;298:587–92.

14. Alonso WJ, Viboud C, Simonsen L, Hirano EW, Daufenbach LZ, Miller MA. Seasonality of influenza in Brazil: a traveling wave from the Amazon to the subtropics. Am J Epidemiol. 2007;165:1434–42. DOI: 10.1093/aje/kwm012
15. Kleinman KP, Abrams A, Mandl K, Platt R. Simulation for assessing statistical methods of biologic terrorism surveillance. MMWR Morb Mortal Wkly Rep. 2005;54(Suppl):101–8.

Address for correspondence: Benjamin J. Cowling, School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Rd, Pokfulam, Hong Kong, People's Republic of China; email: bcowling@hku.hk

Mycobacterium bohemicum and Cervical Lymphadenitis in Children

To the Editor: Members of the genus *Mycobacterium* are well-established causes of granulomatous lymphadenitis in children. *M. bohemicum* was first described in 1998 in a patient with Down syndrome (*1*). The organism is characterized by a unique 16S rRNA gene sequence (*1*) and has been isolated from humans, animals, and the environment (*2*). Published data on *M. bohemicum* are limited to the original species description and 5 additional case reports (*3*–*6*).

We report on 4 cases of cervical lymphadenitis caused by *M. bohemicum* that occurred in 4 children (2 boys, 2 girls) from Austria during 2002–2006. Age range of the children was 2.5–3.5 years (Table). Each child was admitted to the hospital with a history of 2–3 weeks of submandibular swelling that did not respond to oral antimicrobial therapy.

Physical examination showed enlarged cervical lymph nodes under normal skin. Laboratory evaluation showed leukocyte and differential counts within normal limits and a negative result (<2 mg/L) for C-reactive protein. Ultrasonography demonstrated enlarged lymph nodes with heterogeneous echo and a central lacuna-like lesion.

Lymph nodes were excised from each patient. Histologic examination of the nodes demonstrated granulomatous and partly necrotizing inflammation (Table). PCR results for *M. tuberculosis* complex were negative. For 3 patients, the affected lymph nodes were completely removed and no antimicrobial therapy was prescribed. For the other patient, removal of the affected lymph node was incomplete, and clarithromycin and rifampicin for 3 months were prescribed**.** All patients remained healthy for >12 months after therapy.

From all patients, parts of the excised lymph nodes were directly used for acid-fast smear and culture for mycobacteria. All specimens were processed according to national guidelines (the German Institute for Standardization [DIN], Diagnostics for Tuberculosis DIN 58943-3, Cultural Methods for Isolation of Mycobacteria, Berlin: Beuth; 1996.)

Slides for microscopic examination were prepared directly from minced lymph nodes before decontamination, and the tissue was stained according to the Ziehl-Neelsen technique. Each sample was decontaminated, homogenized, and concentrated by using the N-acetyl-L-cysteine-sodium hydroxide method. Samples were spread onto solid slants (Löwenstein-Jensen, Stonebrink; National Reference Center for Mycobacteria, Borstel, Germany) and inoculated into BACTEC Mycobacteria growth indicator tubes (MGIT 960; Becton Dickinson and Company, Cockeysville, MD, USA). Solid slants were incubated at 31°C and 37°C for 8 weeks and inspected for growth weekly. MGITs were incubated in and automatically read with a BACTEC MGIT instrument.

Results of nucleic acid amplification for *M. tuberculosis* complex by using the BD ProbeTec system (Becton Dickinson and Company) as well as the amplification of DNA coding for the mycobacterial 16S rRNA gene were negative. Growth of mycobacteria was detected after 12–17 days in liquid MGIT cultures. For species identification of the cultures, part of the 16S rRNA gene was sequenced (*7*). The resulting sequences were compared with those in international databases (www.ridom.de; www.ncbi.nlm.nih.gov/BLAST). The strains were identified as *M. bohemicum*.

To date, >125 nontuberculous mycobacteria species have been described (www.bacterio.cict.fr/m/mycobacterium.html). Nontuberculous mycobacterial cervical lymphadenitis is most frequently caused by *M. avium* (80%), *M. malmoense*, *M. kansasii, M. lentiflavum*, *M. haemophilum,* and *M. scrofulaceum* (*8*). Because the phenotypic characteristics of *M. bohemicum* closely resemble those of *M. scrofulaceum*, these species can easily be misidentified by analysis of biochemical and cultural features only. The technique by which *M. bohemicum* can clearly be identified is sequence analysis (*8*,*9*).

Other than in the 4 patients reported here, *M. bohemicum* infection has only been reported in 5 patients worldwide (*3*–*6*). Each was a child

Table. Characteristics of 4 children with cervical lymphadenitis caused by *Mycobacterium bohemicum,* Austria, 2002–2006*

Patient no.	Sex; age	Clinical findings	Therapy	Histologic findings
1	M; 2 y, 10 mo	Right submandibular swelling	Incomplete surgical excision; clarithromycin and rifabutin for 3 mo	Granulomatous and partly necrotizing inflammation, multiple giant cells and perinodal fibrosis; no acid-fast bacilli
2†	F; 2 y, 9 mo	Right submandibular swelling	Total lymph node excision	Granulomatous and partly necrotizing inflammation; acid-fast bacilli
3	F; 3 y	Angular right-sided swelling	Total lymph node excision	Granulomatous and partly necrotizing inflammation; sporadic findings of acid-fast bacilli
4	M; 3 y, 7 mo	Swelling in right mandibular area	Total lymph node excision	Granulomatous and partly necrotizing inflammation; no acid-fast bacilli

*All patients had negative PCR results for *Mycobacterium tuberculosis* complex; all patients recovered completely with no relapse.
†Patient had history of psychomotor disorder after perinatal asphyxia.

with laterocervical and submandibular lymphadenitis. Total lymph node excision was performed with a good outcome for all patients except 1, who required additional treatment with antimicrobial drugs because the infected lymph node was incompletely excised (*4*). Additionally, a systemic *M. bohemicum* infection associated with immunodeficiency was reported recently (*10*). Treatment recommendations for nontuberculous mycobacterial lymphadenitis are outlined in discussions of individual nontuberculous mycobacterium species. Guidelines for localized lymphadenitis caused by any nontuberculous mycobacterium species recommend complete surgical excision of the involved lymph nodes (*8*). Additional antimicrobial drug therapy is recommended only for patients for whom removal was incomplete (*8*). Our patient who received combination antimicrobial drug treatment improved, with no relapse.

In summary we report 4 cases of *M. bohemicum* from Austria, a country with 8 million inhabitants. Because these cases were observed in a relatively small country, infections with *M. bohemicum* may be more common than previously thought. More such cases may be discovered as a result of improved microbiologic diagnostic techniques. We believe that *M. bohemicum* should be listed among the species that induce nontuberculous mycobacterial infections.

Julia Huber,* Elvira Richter,† Lothar Binder,‡ Matthias Maaβ,§ Robert Eberl,* and Werner Zenz*

*Medical University of Graz, Graz, Austria; †National Reference Center for Mycobacteria, Borstel, Germany; ‡Elisabethinen Hospital, Linz, Austria; and §University Hospital Salzburg, Salzburg, Austria

DOI: 10.3201/eid1407.080142

References

1. Reischl U, Emler S, Horak Z, Kaustova J, Kroppenstedt RM, Lehn N, et al. *Mycobacterium bohemicum* sp. nov., a new slow-growing scotochromogenic mycobacterium. Int J Syst Bacteriol. 1998;48:1349–55.
2. Torkko P, Suomalainen S, Ivivanainen E, Suutari M, Paulin L, Rudback E, et al. Characterization of *Mycobacterium bohemicum* isolated from human, veterinary, and environmental sources. J Clin Microbiol. 2001;39:207–11. DOI: 10.1128/JCM.39.1.207-211.2001
3. Tortoli E, Kirschner P, Springer B, Bartoloni A, Burrini C, Mantella A, et al. Cervical lymphadenitis due to an unusual mycobacterium. Eur J Clin Microbiol Infect Dis. 1997;16:308–11. DOI: 10.1007/BF01695636
4. Schulzke S, Adler H, Bar G, Heininger U, Hammer J. *Mycobacterium bohemicum*—a cause of paediatric cervical lymphadenitis. Swiss Med Wkly. 2004;134:221–2.
5. Palca A, Aebi C, Weimann R, Bodmer T. *Mycobacterium bohemicum* cervical lymphadenitis. Pediatr Infect Dis J. 2002;21:982–4. DOI: 10.1097/00006454-200210000-00022
6. Tortoli E, Bartoloni A, Manfrin V, Mantella A, Scarparo C, Bottger E. Cervical lymphadenitis due to *Mycobacterium bohemicum.* Clin Infect Dis. 2000;30:210–1. DOI: 10.1086/313600
7. Richter E, Niemann S, Rüsch-Gerdes S, Hoffner S. Identification of *Mycobacterium kansasii* by using a DNA probe (AccuProbe) and molecular techniques. J Clin Microbiol. 1999;37:964–70.
8. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. American Thoracic Society; Infectious Disease Society of America. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007;175:367–416. DOI: 10.1164/rccm.200604-571ST
9. Patel JB, Leonard DG, Pan X, Musser JM. Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 16S rDNA bacterial identification system. J Clin Microbiol. 2000;38:246–51.
10. Glosli H, Stray-Pedersen A, Brun AC, Holtmon LW, Tonjum T, Chapgier A, et al. Infections due to various atypical mycobacteria in a Norwegian multiplex family with dominant interferon-gamma receptor deficiency. Clin Infect Dis. 2008;46:e23–7. DOI: 10.1086/525855

Address for correspondence: Julia Huber, Department of General Pediatrics, Division of Infectiology, Medical University of Graz, Auenbruggerplatz 30, 8036 Graz, Austria; email: julia.huber@meduni-graz.at

Pertussis Surveillance in Private Pediatric Practices, France, 2002–2006

To the Editor: In France, pertussis epidemiology has been extensively studied since 1993. Immunization of children with a highly efficacious pertussis whole-cell (Pw) vaccine (Sanofi Pasteur MSD, Lyon, France) for 40 years (since 1966) has reduced the incidence of pertussis. It has been demonstrated that infectious or vaccinal immunity to pertussis wanes with time and that pertussis is no longer a pediatric disease (*1*–*5*). Transmission now occurs predominantly from adolescents and adults to unvaccinated newborns.

From 1966 through 1995, primary vaccination against pertussis was administered to children at 3, 4, and 5 months of age, and a booster was given at ≈2 years of age. Since 1995, primary vaccination has been administered at 2, 3, and 4 months of age, and a booster is given at 16–18 months of age. Duration of protection of children immunized with Pw vaccine at these schedules is estimated to be ≈7–9 years (*1*,*5*).

In response to the problem of waning immunity, a second pertussis booster immunization at 11–13 years of age was introduced in 1998 (*6*). Development of pertussis acellular (Pa) vaccines has enabled administration of this booster immunization. The French hospital network surveillance system (Renacoq) was established in 1996 to monitor severe pertussis in infants and the effect of late booster immunizations. A cyclic disease pattern was observed; peaks were noted for 1993, 1997, 2000, and 2005. However, the last peak had a low amplitude; since then a diminution in the proportion of siblings who transmitted the infection to young infants was observed (*2*). These results could have been caused

by adolescent booster immunizations.

We evaluated whether the duration of immunity induced by Pw vaccine was still similar to the duration estimated in 1993–1994. This surveillance was necessary because antigenic changes in circulating isolates of *Bordetella pertussis* were observed when compared with vaccine strains (*7*). To achieve this goal, a private pediatric network was set up and data from this surveillance are presented.

From September 2002 through April 2006, 79 pediatricians in France enrolled all infants and children suspected of having pertussis. A standardized data form was completed for age, sex, vaccination data, and source of infection. Biologic confirmation of cases was obtained by using routine laboratory diagnoses, i.e., culture, PCR, or serology. Real-time PCR was performed according to consensus rules (*8*). Routine serodiagnosis was performed by using purified pertussis toxin and Western blotting according to the method of Guiso et al. (*9*) because this is the only diagnostic test free for patients in France. Serologic diagnosis was made by detecting antibodies to pertussis toxin in unvaccinated children or in those vaccinated >1 year earlier. Epidemiologic case-patients were defined as those with a cough for 14 days who had contacts with a confirmed case-patient within 4 weeks of the onset of the cough. No confirmed suspected case-patients had coughs; all were negative for pertussis by biologic diagnosis and did not report contact with a confirmed case-patient.

A total of 383 children were enrolled in the study. However, vaccination status and a biologic diagnosis were available for only 139 children (Table). Forty-seven children had biologically confirmed cases and 92 had nonconfirmed cases. Among children with confirmed cases, only 22 had been vaccinated. At time of disease, the mean ± SD age of these children was 9.9 ± 2.1 years. This age was similar to the age observed during 1993–1994 (*1*,*5*).

The diagnosis for the 92 children suspected of having pertussis was not confirmed biologically. Culture and PCR are used for diagnosis early in the course of pertussis. However, serologic analysis is used later because antibodies are rarely detected before 3 weeks of onset of a cough. More culture and PCR diagnoses were performed for unvaccinated confirmed case-patients than for vaccinated confirmed case-patients. This finding suggests that unvaccinated children were seen by their pediatricians earlier than vaccinated children because the disease was less severe in vaccinated children or that vaccinated children were older than unvaccinated children.

The source of contamination was known for 47% of the confirmed case-patients (Table). This source was either adults (54.4%) or adolescents (41%) who did not receive their second booster immunization or an unvaccinated infant (4.5%). These data are similar to those obtained by the French hospital-based surveillance (*2*). They also support the strategy started in 2004 of recommending a pertussis booster immunization for adults in contact with children and all healthcare workers who come in contact with infants (*10*)

In conclusion, this pediatric surveillance confirms the usefulness of following vaccine recommendations for pertussis and of using biologic techniques to confirm a diagnosis. The vaccine strategy recommending a booster vaccination at 11–13 years of age is still in accordance with epidemiologic features observed. Pediatricians should continue this surveillance to evaluate evolution of *B. pertussis* populations and the effect of replacing Pw vaccines with Pa vaccines.

Acknowledgments

We thank the pediatricians of the Association Clinique et Thérapeutique Infantile du Val de Marne and Association Française de Pédiatrie Ambulatoire networks (C. Abt-Nord, D. Allain, R. Assathiany, I. Aubier, C. Bailly, P. Bakhache, N. Baudino, B. Bedouret, G. Beley, M.

Table. Characteristics of confirmed and nonconfirmed pertussis case-patients for whom vaccination status with pertussis whole-cell (Pw) vaccine was known

	Vaccinated 4× with Pw vaccine (N = 70)		Unvaccinated (N = 69)	
Characteristic	Confirmed case-patients (n = 22)	Nonconfirmed case-patients (n = 48)	Confirmed case-patients (n = 25)	Nonconfirmed case-patients (n = 44)
Diagnostic method				
Culture	0†		3*	
PCR	7†		17*	
Serology	13		3	
Epidemiology	2		2	
Age, y	9.9 ± 2.1†	7.8 ± 3.4	2.3 ± 3.9†	0.8 ± 2.3
p value for age	0.008		0.0046	
Source of contamination				
Adults	5		7	
Adolescents	4		5	
Infants	1 (unvaccinated)		0	

*p = 0.0009 for PCR and culture for case-patients receiving 4 doses of Pw vaccine versus unvaccinated confirmed case-patients.
†p<0.0001 by age for case-patients receiving 4 doses of Pw vaccine versus unvaccinated confirmed case-patients.

Benani, E. Boez, F. Bouillot, C. Bourgin, P. Brichon, J. Cabos, C. Calame-Pilczer, F. Ceccato, J. Cheymol, D. Clavel, C. Copin, F. Corrard, L. Cret, B. De Brito, F. De Grenier, P. Deberdt, A. Delatour, V. Derkx, S. Dieu-Osika, M.N.Domalain, M. Dubosc, S. Duriez, C. Duval, N. El Khoury, A. Elbez, J. Elbhar, M.H. Evroux, E. Fournier-Giorno, D. Garel, A. Gasser, B. Gaudin, S. Gissinger, R. Gorge, J. Guittet, M. Guy, G. Hamelin, S. Hauvespre, A. Hayat, M. Hummel, M. Hunin, M. Koskas, C. Lastmann-Lhami, J.P. Lemaire, L. Levesque, M. Lossouarn, P. Lubelski, N. Maamri, V. Medioni, M.O. Mercier-Oger, M.F. Merklen, P. Migault, A. Napoly, B. Nemesin, J.F. Nicolas, S. Orion, A. Pappo, J. Peguet, C. Petit, M. Pilliot, A. Piollet, V. Poulet-Young, J. Robert, C. Romain, M.C. Rondeau, C. Schlemmer, J.M. Thiron, A. Werner, A. Wollner, C. Wollner, and C.Ythier) for their contributions to this study. We also thank M. Boucherat, L. Langlais, M. Oliveira, S. Tortorelli, and S. Veillault for assistance with the study.

Nicole Guiso,*
France de La Rocque,†
Elisabeth Njamkepo,*
Aurelie Lécuyer,†
Corinne Levy,† Olivier Romain,†
Franck Thollot,‡
Véronique Abitbol,§
Benoit Soubeyrand,¶
Robert Cohen,# and the French Pediatrics Groups Association Clinique et Thérapeutique Infantile du Val de Marne and Association Française de Pédiatrie Ambulatoire

*Institut Pasteur, Paris, France; †Association Clinique et Thérapeutique Infantile du Val de Marne, Saint Maur des Fossés, France; ‡Association Française de Pédiatrie Ambulatoire, Besançon, France; §GlaxoSmithKline, Marly le Roi, France; ¶Sanofi Pasteur MSD, Lyon, France; and #Centre Hospitalier Intercommunal de Créteil, Créteil, France

DOI: 10.3201/eid1407.071246

References

1. Baron S, Njamkepo E, Grimprel E, Begue P, Desenclos JC, Drucker J, et al. Epidemiology of pertussis in French hospitals in 1993 and 1994: thirty years after a routine use of vaccination. Pediatr Infect Dis J. 1998;17:412–8. DOI: 10.1097/00006454-199805000-00013
2. Bonmarin I, Levy-Bruhl D, Baron S, Guiso N, Njamkepo E, Caro V, et al. Pertussis surveillance in French hospitals: results from a 10 year period. Euro Surveill. 2007; [Epub ahead of print].
3. Gilberg S, Njamkepo E, Du Chatelet IP, Partouche H, Gueirard P, Ghasarossian C, et al. Evidence of *Bordetella pertussis* infection in adults presenting with persistent cough in a French area with very high whole-cell vaccine coverage. J Infect Dis. 2002;186:415–8. DOI: 10.1086/341511
4. Wendelboe AM, Njamkepo E, Bourillon A, Floret DD, Gaudelus J, Gerber M, et al. Transmission of *Bordetella pertussis* to young infants. Pediatr Infect Dis J. 2007;26:293–9. DOI: 10.1097/01.inf.0000258699.64164.6d
5. Grimprel E, Begue P, Anjak I, Njamkepo E, Francois P, Guiso N. Long-term human serum antibody responses after immunization with whole-cell pertussis vaccine in France. Clin Diagn Lab Immunol. 1996;3:93–7.
6. Anonymous. Calendrier vaccinal 1998. Avis du conseil supérieur d´hygiène publique de France. Bulletin Epidemiologique Hebdomadaire. 1998;15:61–2.
7. Caro V, Hot D, Guigon G, Hubans C, Arrive M, Soubigou G, et al. Temporal analysis of French *Bordetella pertussis* isolates by comparative whole-genome hybridization *Bordetella pertussis*, Finland and France. Microbes Infect. 2006;8:2228–35. DOI: 10.1016/j.micinf.2006.04.014
8. Riffelmann M, Wirsing von Konig CH, Caro V, Guiso N. Nucleic acid amplification tests for diagnosis of *Bordetella* infections. J Clin Microbiol. 2005;43:4925–9. DOI: 10.1128/JCM.43.10.4925-4929.2005
9. Guiso N, Grimprel E, Anjak I, Begue P. Western blot analysis of antibody responses of young infants to pertussis infection. Eur J Clin Microbiol Infect Dis. 1993;12:596–600. DOI: 10.1007/BF01973637
10. Avis relatif aux recommandations vaccinales contre la coqueluche. Haut Conseil de la Santé Publique. 2008: 1–6 [cited 2008 Apr 14]. Available from http://www.hcsp.fr/hcspi/docspdf/avisrapports/hcspa20080319_coqueluche.pdf

Address for correspondence: Nicole Guiso, Molecular Prevention and Therapy of Human Diseases Unit, National Centre of Reference of Pertussis and other Bordetelloses, Unite de Recherche Associée, Centre National de la Recherche Scientifique 3012, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris CEDEX 15, France; email: nguiso@pasteur.fr

Avian *Mycoplasma lipofaciens* Transmission to Veterinarian

To the Editor: *Mycoplasma* spp. are well-known pathogens in human and veterinary medicine. Mammals, especially primates and including humans, share similar or even identical *Mycoplasma* spp., which might be commensal or pathogenic (*1*). Additionally, sporadic infections of immunocompromised persons with *Mycoplasma* spp. that originated from domestic animals have been reported (*1*); susceptibility in this human population is increased (*2*,*3*). *M. phocicerebrale* is the only *Mycoplasma* pathogen of animals that regularly infects humans, causing a disease called seal fingers (*1*,*4*). However, we report a human infection with an avian *Mycoplasma* organism.

A clinical trial to investigate the capability of *M. lipofaciens* (strain ML64) (*5*) to spread horizontally between infected and noninfected turkey poults in an incubator demonstrated airborne transmission of the pathogen within 24 hours (*6*). During the trial, the veterinarian conducting the study, a 36-year-old man, was monitored for infection. Each day, 2 swabs were taken from both nostrils, starting

from the day before the infected poults hatched (day 0) through day 7 after the poult hatching date. When handling eggs and poults, the veterinarian wore gloves but not a protective mask. Two days after the poults hatched (day 3), the veterinarian reported throat pain and a slight rhinitis, which indicated a respiratory disease. The next day only the rhinitis with minor nasal pain was present.

One nasal swab from each sampling day was used for *Mycoplasma* spp. culture (*5*). Isolated *Mycoplasma* organisms were subjected to an immunobinding assay (*6*) with antiserum against *M. lipofaciens*, *M. buteonis*, *M. falconis*, *M. gypis*, *M. gallisepticum*, *M. meleagridis*, *M. synoviae*, and *M. iowae*, selected because the veterinarian regularly handled these isolates. The second nasal swab from each sampling day was taken to detect *Mycoplasma* DNA by PCR (*5*). The samples for PCR testing were stored at –20°C and tested only when attempts to isolate *Mycoplasma* spp. failed.

Six weeks after the infection, a serum sample from the patient was examined for specific antibodies against *M. lipofaciens* by 3 different methods: modified immunobinding assay (*6*) (which used patient's serum and peroxidase-conjugated goat-antihuman serum [STAR90P, Serotec Ltd, Oxford, UK]), a growth inhibition test, and antibody titers of serum dilutions. The first 2 methods used *M. lipofaciens* (strain ML64) from a turkey trial (*6*) and the reference strains of *M. buteonis* (Bb/T2g) and *M. falconis* (H/T1). For the third method, to determine the titer, 2-fold serial dilutions of the patient's serum samples were prepared and incubated with a bacterial suspension (*M. lipofaciens* strain ML64) containing 3.2×10^2 CFU. The antibody titer was based on the highest serum dilution capable of reducing 50% of the mean CFU count.

Before the infected poults hatched, attempts to isolate *Mycoplasma* organisms and demonstrate *Mycoplasma* DNA from the nasal swabs were unsuccessful. However, from the day of hatching (day 1) until 3 days later (day 4), *Mycoplasma* organisms were isolated from the nasal swabs and identified as *M. lipofaciens*. On day 5, only *Mycoplasma* DNA was demonstrated (Table). Specific antibodies against *M. lipofaciens* (strain ML64) were detected by using the immunobinding assay and growth inhibition test. Antibodies against other *Mycoplasma* spp. were not detected. Antibody titer against *M. lipofaciens* was 128.

M. lipofaciens have been reported from a chicken, turkey, duck (*7*,*8*), and a raptor egg (*5*). Strain ML64 is highly pathogenic for chicken and turkey embryos and can be transmitted by air (*6*,*9*). The veterinarian handling the infected poults was free of nasal *Mycoplasma* organisms a day before contact. His infection occurred concurrent with demonstration of airborne transmission among poults. The isolation of *M. lipofaciens* from his nares for 4 days demonstrates the infectivity and reproductive capability of this *Mycoplasma* strain in humans: as a pure contaminant, isolation for several days would be unlikely. Christensen et al. (*10*) have reisolated different avian *Mycoplasma* strains (*M. gallisepticum*, *M. synoviae*, *M. iowae*) from a human nose, from 12 hours through 1 day after artificial infection, demonstrating differences between avian strain abilities to survive on human mucosa. *M. lipofaciens* invasiveness for humans is underscored by finding specific antibodies against this species 6 weeks after infection. Cross-reactivity to other *Mycoplasma* spp. cannot be excluded but seems unlikely.

This study suggests that *M. lipofaciens* (strain ML64) can be transmitted successfully to humans and may cause clinical symptoms; the study documents nonartificial human infection with an avian *Mycoplasma* sp. These findings should be considered especially for humans highly susceptible to *Mycoplasma* infections, including children and persons with congenital or acquired immunodeficiencies (*2*,*3*).

Acknowledgment

We thank Janet Bradbury for providing the *Mycoplasma* antiserum used in the immunobinding assay as well as the reference strains used.

Michael Lierz,*
Andreas Jansen,†
and Hafez M. Hafez*

*Free University of Berlin, Berlin, Germany; and †Robert Koch Institute, Berlin

DOI: 10.3201/eid1407.071703

References

1. Pitcher DG, Nicholas RAJ. *Mycoplasma* host specificity: Fact or fiction? Vet J. 2005;170:300–6. DOI: 10.1016/j.tvjl.2004.08.011
2. Roifman CM, Rao CP, Lederman HM, Lavi S, Quinn P, Gelfand EW. Increased susceptibility to *Mycoplasma* infection in patients with hypogammaglobulinaemia. Am J Med. 1986;80:590–4. DOI: 10.1016/0002-9343(86)90812-0

Table. Timeline of natural infection of veterinarian with *Mycoplasma lipofaciens* (strain ML64) from infected poults*

	Day							
Finding	0	1†	2	3	4	5	6	7
Isolation	–	*M. l.*	*M. l.*	*M. l.*	*M. l.*	–	–	–
PCR result‡	–	ND	ND	ND	ND	+	–	–
Clinical signs	None	None	None	Throat pain, slight rhinitis	Slight rhinitis, nasal pain	None	None	None

*–, negative; *M.l., Mycoplasma lipofaciens* (identified by immunobinding assay); ND, not done; +, positive.
†Hatching of infected poults and demonstration of aerosol transmission among poults.
‡Detection of mycoplasma DNA per Lierz et al. (*5*).

3. Baseman JB, Tully JG. *Mycoplasma:* sophisticated, reemerging, and burdened by their notoriety. Emerg Infect Dis. 1997;3:21–32.
4. Baker AS, Ruoff KL, Madoff S. Isolation of *Mycoplasma* species from a patient with seal finger. Clin Infect Dis. 1998;27:1168–70. DOI: 10.1086/514980
5. Lierz M, Hagen N, Harcourt-Brown N, Hernandez-Divers SJ, Lueschow D, Hafez HM. Prevalence of mycoplasmas in eggs from birds of prey using culture and a genus-specific mycoplasma polymerase chain reaction. Avian Pathol. 2007;36:145–50. DOI: 10.1080/03079450701213347
6. Lierz M, Deppenmeier S, Gruber AD, Brokat S, Hafez HM. Pathogenicity of *Mycoplasma lipofaciens* strain ML64 for turkey embryos. Avian Pathol. 2007;36:389–93. DOI: 10.1080/03079450701589126
7. Bradbury JM, Forrest M, Williams A. *Mycoplasma lipofaciens*, a new species of avian origin. Int J Syst Bacteriol. 1983;33:329–35.
8. Bencina D, Dorrer D, Tadina T. *Mycoplasma* species isolated from six avian species. Avian Pathol. 1987;16:653–64. DOI: 10.1080/03079458708436413
9. Lierz M, Stark R, Brokat S, Hafez HM. Pathogenicity of *M. lipofaciens* strain ML64, isolated from an egg of a Northern Goshawk (*Accipiter gentilis*), for chicken embryos. Avian Pathol. 2007;36:151–3. DOI: 10.1080/03079450701213354
10. Christensen NH, Yavari CA, McBai AJ, Bradbury JM. Investigations into the survival of *Mycoplasma gallisepticum, Mycoplasma synoviae* and *Mycoplasma iowae* on materials found in the poultry house environment. Avian Pathol. 1994;23:127–43. DOI: 10.1080/03079459408418980

Address for correspondence: Michael Lierz, Institute for Poultry Diseases, Free University of Berlin, Königsweg 63, 14163 Berlin, Germany; email: lierz.michael@vetmed.fu-berlin.de

Urinary Tract Infection Caused by Capnophilic *Escherichia coli*

To the Editor: Increased atmospheric CO_2 concentrations promote the growth of fastidious microorganisms. However, the possibility that a strain of *Escherichia coli* can be CO_2 dependent is exceptional (*1*).

An isolate of capnophilic *E. coli* was responsible for a urinary tract infection (UTI) in a 77-year-old woman at the University Hospital of Guadalajara (Spain) in November 2002. Urine was cultured on a cystine-lactose-electrolyte-deficient agar plate and incubated at 37°C in an atmosphere containing 6% CO_2 for 1 day. After 24 hours, the culture yielded gram-negative rods (>10^5 CFU/mL) in pure culture. The organism was motile, catalase positive, and oxidase negative. The strain could not be identified by using the MicroScan WalkAway-40 system (DadeBerhing, Inc., West Sacramento, CA, USA). A subculture was performed, and the organism did not grow on sheep blood agar and MacConkey agar plates at 37°C in ambient air. However, a subculture incubated at 37°C for 24 hours in an atmosphere of 6% CO_2 produced smooth colonies 2–3 mm in diameter on sheep blood agar and MacConkey agar plates. The organism fermented lactose, and the indole reaction (BBL DrySlidet, Becton Dickinson Co., Sparks, MD, USA) performed on sheep blood agar was negative. The strain grew well on Schaedler agar plates after anaerobic incubation for 48 hours. The isolate remained capnophilic after 5 subcultures. The strain was identified as *E. coli* by using the Biolog GN2 panel (Biolog, Inc., Hayward, CA, USA) (100%, T = 0,534), after incubation of the panel in an atmosphere containing 6% CO_2 for 1 day. The API 20E system (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions without $C0_2$ incubation also identified *E. coli* (profile 5004512). The identification was confirmed by means of 16S rDNA sequence analysis (1,472 bp obtained by PCR amplification by a previously reported method [*2*]), which showed 99% similarity with *E. coli* sequence (GenBank accession no. CP000802). The 16S sequence showed similarity with *Shigella* species; however, this identification was not considered because the strain fermented lactose on MacConkey agar and agglutinations with *Shigella* antiserum were negative. The original 16S rDNA sequence was deposited in GenBank (accession no. EU555536).

The antimicrobial drug susceptibility profile was determined by incubating Mueller-Hinton agar plates at 37°C in an atmosphere containing 6% CO_2 by the disk diffusion method, according to National Committee for Clinical Laboratory Standards recommendations (*3*). The isolate was susceptible to ampicillin, amoxicillin/clavulanic acid, piperacillin, cefazolin, cefuroxime, cefotaxime, nitrofurantoin, fosfomycin, trimethoprim-sulfamethoxazole, gentamicin, tobramycin, amikacin, norfloxacin, and ciprofloxacin. MICs were obtained for the following antimicrobial agents with the E-test method (AB Biodisk, Solna, Sweden), performed on Mueller-Hinton agar plates incubated in a 6% CO_2 atmosphere: ampicillin (1.5 μg/mL), amoxicillin (3 μg/mL), cefotaxime (0.064 μg/mL), imipenem (0.094 μg/mL), piperacillin (2 μg/mL), and ciprofloxacin (0.008 μg/mL).

E. coli is the most common pathogen among patients with uncomplicated UTIs (*4*). Two cases of UTIs due to carbon dioxide–dependent strains of *E. coli* have been reported (*1*). The mechanisms for development of CO_2 dependence are unknown (*5*). CO_2 can play a role in the growth of *E. coli* as a substrate for carboxylation reactions (*6*). Other members of the family *En-*

terobacteriaceae (such as some strains of *Klebsiella* spp.) and other organisms (such as *Staphylococcus aureus*), can have similar requirements (*7*,*8*).

There is not 1 best way of performing urine cultures. Guidelines for the diagnosis of UTI includes the use of sheep blood agar and either MacConkey agar or a similar selective medium for routine urine culture. The plates should be incubated overnight (at least 16 hours) at 37°C in ambient air; alternatively, the blood agar plate can be incubated in elevated (3%–8%) CO_2 (*9*). For fastidious microorganisms, chocolate agar can be added to the MacConkey agar and the plates incubated in 5% CO_2 for 2 days (*9*).

The real incidence of these infections is unknown, but the rarity of these strains suggests that the incidence is low. However, the real incidence of UTI caused by capnophilic *E. coli* may be underestimated because urine cultures are not usually incubated in CO_2. In addition, urine cultures are not performed for many women with uncomplicated cystitis. Other fastidious uropathogens such as *Haemophilus influenzae* and *H. parainfluenzae*, also require special media and incubation in an atmosphere of CO_2 (*9*). The low frequency of these strains suggests that incubation of routine urine cultures in an atmosphere containing CO_2 is not necessary. Incubation in CO_2 should be ordered only if the patient has pyuria and a previous negative urine culture after incubation in ambient air or if the patient is unresponsive to empiric therapy and routine urine culture is negative. Good clinician–laboratory communication is vital. Further studies should be performed to ascertain the real incidence of UTIs caused by capnophilic strains of *E. coli.*

Because no breakpoints are available for antimicrobial agents against capnophilic strains of *E. coli*, we used published interpretative criteria or *Enterobacteriaceae* (*3*). The strain was susceptible to all antimicrobial agents that we tested. The impact of CO_2 on the susceptibility of capnophilic strains of *E. coli* is unknown. Susceptibility of some antimicrobial agents such as quinolones can be influenced by the pH change and enhanced growth that occur during CO_2 incubation when testing capnophilic organisms (*10*).

Daniel Tena,*
Alejandro González-Praetorius,*
Juan Antonio Sáez-Nieto,†
Sylvia Valdezate,†
and Julia Bisquert*

*University Hospital of Guadalajara, Guadalajara, Spain; and †Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

DOI: 10.3201/eid1407.071053

References

1. Eykyn S, Phillips I. Carbon dioxide-dependent *Escherichia coli.* BMJ. 1978;1:576.
2. Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacteria isolates. J Clin Microbiol. 2000;38:3623–30.
3. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests. Approved standard M2–A8. Wayne (PA): The Committee; 2003.
4. Kahlmeter G. An international survey of the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infection: the ECO.SENS Project. J Antimicrob Chemother. 2003;51:69–76. DOI: 10.1093/jac/dkg028
5. Repaske R, Clayton MA. Control of *Escherichia coli* growth by CO_2. J Bacteriol. 1978;135:1162–4.
6. Kozliak EI, Fuchs JA, Guilloton MB, Anderson PM. Role of bicarbonate/CO_2 in the inhibition of *Escherichia coli* growth by cyanate. J Bacteriol. 1995;177:3213–9.
7. Barker J, Brookes G, Johnson T. Carbon dioxide–dependent *Klebsiellae.* BMJ. 1978;1:300.
8. Rahman M. Carbon dioxide–dependent *Staphylococcus aureus* from abscess. BMJ. 1977;2:319.
9. Clarridge JE, Johnson JR, Pezzlo MT. Laboratory diagnosis of urinary tract infections. In: Weissfeld AS, editor. Cumitech 2B. Washington: ASM Press; 1988. p. 2–19.
10. Bolmstrom A, Karlsson A. Influence of CO_2 incubation on quinolone activity against *Streptococcus pneumoniae* and *Haemophilus influenzae.* Diagn Microbiol Infect Dis. 2002;42:65–9. DOI: 10.1016/S0732-8893(01)00338-8

Address for correspondence: Daniel Tena, Section of Microbiology, University Hospital of Guadalajara, Calle Donantes de Sangre s/n 19002 Guadalajara, Spain; email: danielt@sescam.jccm.es

Duck Migration and Past Influenza A (H5N1) Outbreak Areas

To the Editor: In 2005 and 2006, the highly pathogenic avian influenza (HPAI) virus subtype H5N1 rapidly spread from Asia through Europe, the Middle East, and Africa. Waterbirds are considered the natural reservoir of low pathogenic avian influenza viruses (*1*), but their potential role in the spread of HPAI (H5N1), along with legal and illegal poultry and wildlife trade (*2*), is yet to be clarified.

The garganey (*Anas querquedula*) is the most numerous duck migrating between Eurasia and Africa: ≈2 million gather in the wetlands of Western Africa every northern winter (*3*). We report on a spatial correlation between the 2007 migration path of a garganey monitored through satellite telemetry and areas that had major HPAI (H5N1) outbreaks from 2005 through 2007.

Seven garganeys were captured, sampled, and fitted with a 12-g satellite transmitter in northern Nigeria (Hadejia-Nguru Wetlands; 12°48′N; 10°44′E) in the period February 7–15, 2007. All cloacal and tracheal swabs tested negative for avian influenza virus by real-time reverse transcription–PCR analysis of the matrix gene. One second-year (>9-month-

old) female garganey migrated from northern Nigeria to Russia in April–May 2007 (online Appendix Figure, available from www.cdc.gov/EID/content/14/7/1164–appG.htm), where she remained until the end of July. During this 6-week spring migration over the Sahara Desert, Mediterranean Sea, and Eastern Europe, this duck stopped at 3 main stopover sites in Crete, Turkey (Bosphorus region), and Romania (Danube River delta). The duck migrated back to the Danube delta in August, where it remained until November, when the signal was lost. Other garganeys we monitored stopped transmitting before initiating spring migration (n = 3) or remained in West Africa during spring and summer (n = 3), which suggests a stress linked to capture or constraint from the transmitter attachment.

This transcontinental migration path connects several areas of past major HPAI (H5N1) outbreaks (online Appendix Figure). The wintering area in Nigeria where this duck was caught and remained for 8 weeks before spring migration is located where a large number of outbreaks have occurred repeatedly since February 2006 (the closest being 30 km away). This bird reached its breeding ground in Russia near Moscow and stayed for 2 months in an area that had several outbreaks in backyard poultry in February 2007 (the closest being 30 km away). Finally, the Danube delta, used as a resting ground for 3 months in late summer and autumn, is also an area with recurring outbreaks since October 2005 in wild and domestic birds, with the most recent case reported in November 2007 (the closest being 10 km away). The initial spread of HPAI virus (H5N1) from Eurasia to Africa occurred in autumn and winter 2005–06. The migratory movements we observed during spring and summer in this study were not temporally correlated with any reported HPAI (H5N1) outbreak, either in sequence or period; hence, they should not be interpreted as evidence of the role of wild bird in expansions of the virus.

During spring migration from Nigeria to Russia, the garganey stopped several days in wetlands situated close to areas of past outbreaks in the Danube delta (4 days at a distance of 1–4 km from October 2005 outbreaks) and Lake Kus, Turkey (8 days at a distance of 10–30 km from October 2005 outbreaks). The occurrence of past outbreaks indicates that the duck used wetlands favorable to HPAI virus (H5N1) transmission as stopover sites. The relatively long stopover periods enabled prolonged contact of migratory ducks with local domestic and wild bird populations or through shared water, thus prolonging the potential for virus transmission. Considering the persistence of infectivity of HPAI virus (H5N1) in aquatic habitats (*4*), the number of migratory ducks congregating at stopover sites from various geographic origins and destinations, and the asynchronous timing of the arrival and departure of migratory ducks (*5*), we believe that these sites may provide locations for disease transmission and possible spread upon movement of wild birds.

The satellite-fitted female garganey covered distances between stopover sites of >2,000 km in <2 days, traveling at an estimated speed of 60 km/h. This large-scale movement in a short period, coupled with experimental exposure trials demonstrating viral shedding of up to 4 days in ducks with no clinical signs of infection (*6*), is consistent with potential viral transmission over great distances.

These facts illustrate how a pathogen such as HPAI virus (H5N1) can potentially be transported rapidly by migratory birds across continents. However, the physiologic impact of an HPAI (H5N1) infection on the ability of birds to migrate long distances is still unknown (*7*) and to date, most empirical evidence suggests that wild birds have only moved short distances (a few hundred kilometers) likely carrying HPAI virus (H5N1) (*8*). Despite extensive global wildlife surveillance efforts and with the exception of a few reported cases of HPAI (H5N1) infection in apparently healthy wild ducks (*9*,*10*), evidence of wild bird involvement in the spread of HPAI virus (H5N1) over long distances is still lacking.

Acknowledgments

We thank the conservator-general of Nigerian National Parks for permission to capture birds for telemetry study in Nigeria, and ornithologists from the A.P. Leventis Ornithological Research Institute, the Ottenby Bird Observatory, Bird Studies Canada, and the Istituto Nazionale per la Fauna Selvatica. We also thank the Zoo of Lunaret (Montpellier, France) for allowing us to test the harness-attached transmitter on captive garganeys.

Financial support (OSRO/GLO/604/UK) for this project was provided by the Government of the United Kingdom of Great Britain and Northern Ireland, represented by the Department for International Development.

Nicolas Gaidet,*
Scott H. Newman,†
Ward Hagemeijer,‡
Tim Dodman,‡ Julien Cappelle,*
Saliha Hammoumi,*
Lorenzo De Simone,†
and John Y. Takekawa§

*Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Montpellier, France; †Food and Agriculture Organization of the United Nations, Rome, Italy; ‡Wetlands International, Wageningen, the Netherlands; and §US Geological Survey, Vallejo, California, USA

DOI: 10.3201/eid1407.071477

References

1. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza a virus in wild birds. Science. 2006;312:384–8. DOI: 10.1126/science.1122438

2. Kilpatrick AM, Chimura AA, Gibbons DW, Fleischer RC, Marra PP, Daszak P. Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci U S A. 2006;103:19368–73. DOI: 10.1073/pnas.0609227103
3. Delany S, Scott D, eds. Waterbird population estimates. 4th ed. Wageningen (the Netherlands): Wetlands International; 2006.
4. Brown JD, Swayne DE, Cooper RJ, Burns RE, Stallknecht DE. Persistence of H5 and H7 avian influenza viruses in water. Avian Dis. 2007;51(Suppl):285–9. DOI: 10.1637/7636-042806R.1
5. Guillemain M, Fritz H, Klaassen M, Johnson AR, Hafner H. Fuelling rates of garganey (*Anas querquedula*) staging in the Camargue, southern France, during spring migration. J Ornithol. 2004;145:152–8. DOI: 10.1007/s10336-004-0026-9
6. Brown JD, Stallknecht DE, Beck JR, Suarez DL, Swayne DE. Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. Emerg Infect Dis. 2006;12:1663–70.
7. Weber TP, Stilianakis NI. Ecologic immunology of avian influenza (H5N1) in migratory birds. Emerg Infect Dis. 2007;13:1139–43.
8. Watch EMPRES. HPAI in Europe 2007: concurrent outbreaks in poultry and wild birds. Rome: Food and Agriculture Organization of the United Nations; 2007 [cited 2007 Sep 12]. Available from www.fao.org/docs/eims/upload/231765/EW_Europe_aug07_ai.pdf
9. Saad MD, Ahmed LS, Gamal-Eldein MA, Fouda MK, Khalil FM, Yingst SL. Possible avian influenza (H5N1) from migratory bird, Egypt. Emerg Infect Dis. 2007;13:1120–1.
10. Chen H, Li KS, Wang J, Fan XH, Rayner JM, Vijaykrishna D, et al. Establishment of multiple sublineages of H5N1 influenza virus in Asia: Implications for pandemic control. Proc Natl Acad Sci U S A. 2006;103:2845–50. DOI: 10.1073/pnas.0511120103

Address for correspondence: Nicolas Gaidet, Département Environnements et Sociétés, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, UR Gestion Intégrée de la Faune TA 30/E Campus International de Baillarguet, 34398 Montpellier, France; email: nicolas.gaidet@cirad.fr

Dihydrofolate Reductase I164L Mutation in *Plasmodium falciparum*, Madagascar

To the Editor: Malaria remains a major public health problem and a primary cause of illness in Madagascar (*1*). Since 2005, the National Malaria Control Program has revised its treatment policy and replaced chloroquine (CQ) with artesunate plus amodiaquine as first-line therapy for uncomplicated malaria and CQ with sulfadoxine-pyrimethamine (SP) for prevention of malaria during pregnancy. The latter choice was partially supported by high effectiveness of SP and absence of pyrimethamine resistance in Madagascar, in contrast to proximal African countries such as the Comoros Islands (*2*,*3*).

Analysis of the molecular basis of antimalarial drug resistance has demonstrated that mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase genes are associated with development of SP resistance. It has been assumed that pyrimethamine resistance conferred by multiple mutations arose through stepwise selection of the S108N single mutant (except for the A16V/S108T allele). This single-point mutation decreases the sensitivity of *dhfr* to pyrimethamine in vitro by ≈10× (*4*). Subsequent mutations, such as N51I and C59R, cause additional decreases in the sensitivity of *dhfr* to pyrimethamine. Parasites with a triple-mutant allele (51I/59R/108N) are less sensitive to pyrimethamine in vitro, and patients infected with these parasites have a high probability of not responding to SP treatment (*5*).

Addition of I164L to 51I/59R/108N creates a quadruple-mutant allele and decreases the sensitivity of *dhfr* by ≈1,000× (*4*), eliminating the clinical effectiveness of SP, as observed in Southeast Asia and South America. However, the situation in Africa seems to be different because most studies conducted since the mid 1990s have shown the quadruple mutant to be rare, even in areas of intensive pyrimethamine use (*6*). Increasing SP resistance is principally a result of rapid selection for parasites that carry a triple-mutant allele that arose in Southeast Asia and has spread widely in Africa (*7*,*8*).

In 2006, blood samples were obtained from 114 children 6 months to 15 years of age enrolled in a clinical trial monitoring the efficacy of SP in treatment of uncomplicated *Plasmodium falciparum* malaria. The *dhfr* gene from pretreatment samples was sequenced at the Genomics Platform of the Pasteur Institute in Paris, France. Four (3%) samples contained the 108N single-mutant allele, 37 (32%) contained the 51I/59R/108N triple-mutant allele, and 1 (<1%) contained the I164L single-mutant allele. This latter allele was obtained from the blood of a 15-year-old girl from Ejeda in southern Madagascar. At enrollment in the trial, she had an axillary temperature of 37.8°C and a *P. falciparum* asexual parasite count of 74,880/μL. She was treated with the standard SP regimen (25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine as a single dose on day 0). On the basis of the World Health Organization 2003 protocol (*9*), early treatment failure was noted on day 2, when the patient had signs of malaria with a temperature of 40°C and a parasite count of 770/μL. She was successfully retreated with a rescue regimen (quinine, 8 mg base/kg, 3 times a day for 7 days).

To confirm detection of the I164L allele, parasite DNA was extracted from blood spots obtained on days 0, 1, and 2 and sequenced. DNA templates were sent to a second independent laboratory (Department of Genome Sciences, University of Washington, Seattle, WA, USA) to rule out misiden-

tification or polymerase errors. Nucleotide sequences obtained were compared with wild-type sequence (isolate 3D7, pyrimethamine-sensitive, GenBank accession no. NC_004318.1) by using BioEdit software (www.mbio.ncsu.edu/BioEdit/BioEdit.html). No point mutations other than I164L were observed in any samples obtained on 3 consecutive days. The nucleotide sequence determined in this study has been deposited in the GenBank database (accession no. EU280750).

Our report provides new insights into development of antifolate-resistant malaria and supports the findings of McCollum et al. (*10*) that the mutations in the *dhfr* gene do not always occur in a predictable, ordered, stepwise manner. Although the I164L allele was detected in a clinical sample from a patient who showed early treatment failure, blood levels of the drug were not measured. Thus, it is not possible to establish a causal connection between the I164L mutation and SP resistance. Moreover, the substantial reduction in parasite counts observed on days 0, 1, and 2 implied that SP treatment helped reduce parasite counts.

Our data confirm that the I164L mutation is beginning to appear in Africa. This observation highlights the risk for emergence and spread of the 51I/59R/108N/164L quadruple-mutant *dhfr* genotype by recombination between the I164L genotype and the 51I/59R/108N triple-mutant genotype, which would make SP widely ineffective for intermittent treatment of malaria during pregnancy. Further studies are needed to understand whether the I164L genotype has emerged in Madagascar because of local evolutionary history or has been introduced by gene flow from India or Southeast Asia, and to evaluate the risk for spread of this genotype to the rest of Africa through Madagascar.

Acknowledgments

We thank the patients and their parents or guardians for participating in the clinical trial monitoring antimalarial drug resistance; the clinical study team in Ejeda (Diamondra Raveloariseheno, Brunette Razanadrazanina, and M. Leo Paul Andriamahazo) for assistance; and health workers at the health centers of Ejeda, their respective district administrations, and the staff of the Ministry of Health of Madagascar for enabling us to conduct the study.

This study was supported by grants from Natixis/Impact Malaria through the Observatoire de la Résistance aux Antipaludiques project and the Genomics Platform, Pasteur Génopôle, Pasteur Institute, France. Collection of samples was supported by the global fund project for Madagascar round 3 (Community Action to Roll Back Malaria grant no. MDG-304 -G05-M).

Didier Ménard,*
Valérie Andriantsoanirina,*
Martial Jahevitra,*
Céline Barnadas,*
Magali Tichit,†
Christiane Bouchier,†
and Carol Hopkins Sibley‡

*Institut Pasteur, Antananarivo, Madagascar; †Institut Pasteur, Paris, France; and ‡University of Washington, Seattle, Washington, USA

DOI: 10.3201/eid1407.071498

References

1. Institut National de la Statistique (INSTAT) (Madagascar) et ORC Macro. Enquête démographique et de santé, Madagascar 2003–2004: rapport de synthèse. Calverton (MD): INSTAT et ORC Macro; 2005.
2. Randrianarivelojosia M, Ariey F, Raharimalala LA, Parzy D, Rogier C, Jambou R. Current absence of pyrimethamine resistance of *Plasmodium falciparum* in Madagascar. Trans R Soc Trop Med Hyg. 2002;96:557–79. DOI: 10.1016/S0035-9203(02)90441-4
3. Parola P, Pradines B, Simon F, Carlotti MP, Minodier P, Ranjeva MP, et al. Antimalarial drug susceptibility and point mutations associated with drug resistance in 248 *Plasmodium falciparum* isolates imported from Comoros to Marseille, France in 2004 2006. Am J Trop Med Hyg. 2007;77:431–7.
4. Hastings MD, Bates SJ, Blackstone EA, Monks SM, Mutabingwa TK, Sibley CH. Highly pyrimethamine-resistant alleles of dihydrofolate reductase in isolates of *Plasmodium falciparum* from Tanzania. Trans R Soc Trop Med Hyg. 2002;96:674–6. DOI: 10.1016/S0035-9203(02)90349-4
5. Gregson A, Plowe CV. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev. 2005;57:117–45. DOI: 10.1124/pr.57.1.4
6. Nzila A, Ochong E, Nduati E, Gilbert K, Winstanley P, Ward S, et al. Why has the dihydrofolate reductase 164 mutation not consistently been found in Africa yet? Trans R Soc Trop Med Hyg. 2005;99:341–6. DOI: 10.1016/j.trstmh.2004.07.002
7. Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T. Intercontinental spread of pyrimethamine-resistant malaria. Science. 2004;305:1124. DOI: 10.1126/science.1098876
8. Noranate N, Durand R, Tall A, Marrama L, Spiegel A, Sokhna C, et al. Rapid dissemination of *Plasmodium falciparum* drug resistance despite strictly controlled antimalarial use. PLoS One. 2007;2:e139. DOI: 10.1371/journal.pone.0000139
9. World Health Organization. Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria. Geneva: The Organization; 2003.
10. McCollum AM, Poe AC, Hamel M, Huber C, Zhou Z, Shi YP, et al. Antifolate resistance in *Plasmodium falciparum*: multiple origins and identification of novel *dhfr* alleles. J Infect Dis. 2006;194:189–97. DOI: 10.1086/504687

Address for correspondence: Didier Ménard, Institut Pasteur, BP 1274, Antananarivo 101, Madagascar; email: dmenard@pasteur.mg

Bartonella quintana and *Coxiella burnetii* as Causes of Endocarditis, India

To the Editor: In industrialized countries, blood culture is negative for 2.5%–31% of infectious endocarditis cases (*1*). In developing countries such as South Africa (*2*), Algeria (*3*), and Pakistan (*4*), culture is negative for 48% to 56%. Negative cultures delay diagnosis and treatment, which profoundly affects clinical outcome. Negative blood cultures commonly result from previous administration of antimicrobial drugs, right-sided endocarditis, or fastidious or noncultivable pathogens (*1*). Our aim was to identify fastidious agents of blood culture–negative endocarditis by serology**.** Because of recent attention to zoonotic microorganisms as agents of this condition in developing countries (*1*), we investigated the prevalence of *Coxiella burnetii*, *Bartonella* spp., and *Brucella melitensis* among endocarditis patients in India.

We cultured blood from 111 patients admitted to the Government General Hospital, Chennai, India, from August 2005 through December 2006, with a diagnosis of infectious endocarditis according to the Duke criteria (*5*). Informed consent was obtained from all patients. Three blood samples from each patient, collected at hourly intervals, were inoculated into brain–heart infusion broth supplemented with 0.04% sodium polyanethol sulfonate (HiMedia, Mumbai, India). Cultures were incubated at 37°C in a 5% CO_2 atmosphere for 14 days and checked each day for turbidity. Subcultures were made on 5% sheep blood and MacConkey agar at 37°C at 24 hours, 48 hours, and when culture broth appeared turbid.

Blood cultures were negative for 80 (72%) of the 111 patients. Serum from 63 patients was available for serologic testing. Of these patients, 30 were male and 33 were female; age range was 5–65 years and mean age was 25.5 years. Endocarditis involved the native valve for 60 (95.23%) and a prosthetic valve for 3 (4.76%). The most frequent predisposing factor was rheumatic heart disease, found in 38 (60.31%). Of the 60 with native valve endocarditis, the involved valve was mitral for most (36, 60.0%), followed by aortic (8, 13.33%), tricuspid (7, 11.66%), and pulmonary (1, 1.66%); 8 (13.33%) had both valvular and nonvalvular endocarditis. Of the 3 patients with prosthetic valve endocarditis, the involved valve was mitral for 2 and aortic for 1.

Serum samples were screened for *Bartonella* spp. and *C. burnetii* by microimmunofluorescence (*6*,*7*). A diagnosis of endocarditis was based on an immunoglobulin (Ig) G titer ≥800 to phase I *C. burnetii*; this titer has a positive predictive value of 98% (*6*). A diagnosis of *Bartonella* infection was based on the combination of a positive microimmunofluorescence titer (IgG to *B. quintana* or *B. henselae* ≥200) and a Western blot profile consistent with endocarditis (*8*).

Identification of the causative species was obtained by Western blot after cross-adsorption with either *B. henselae* or *B. quintana* antigens (*8*).

Table. Clinical findings and causative agent for 9 patients with blood culture–negative endocarditis, India, August 2005–December 2006*

Patient age, y/sex	Underlying cardiac condition	Echocardiographic findings	IgG titer to *Bartonella* spp.	IgG titer to *Coxiella burnetii* phase I	Causative agent
25/F	Right atrium fistula	Vegetation attached to tricuspid valve	400	100	*Bartonella quintana*
46/M	Rheumatic heart disease	Vegetation attached to anterior mitral leaflet	0	800	*Coxiella burnetii*
14/M	Rheumatic heart disease	Vegetation attached to tip of anterior mitral leaflet	200	0	*B. quintana*
13/M	Rheumatic heart disease	Vegetation attached to anterior mitral leaflet	200	0	*B. quintana*
28/M	Bicuspid aortic valve disease	Vegetation attached to anterior coronary cusp of aortic valve	400	0	*B. quintana*
30/M	Rheumatic heart disease	Vegetation attached to both anterior and posterior mitral leaflet extending to chordae tendinae	200	0	*B. quintana*
50/F	Rheumatic heart disease	Vegetation attached to noncoronary cusp of aortic valve	400	0	*Bartonella* spp.
40/M	Bicuspid aortic valve disease	Calcified aortic valve	400	0	*B. quintana*
40/M	Double chamber right ventricle and subaortic perimembranous ventricular septal defect	Vegetation attached to right atrium anterior leaf of tricuspid valve and lateral cusp of pulmonary valve	800	0	*B. quintana*

*Ig, immunoglobulin.

Antibodies to *B. melitensis* were detected by agglutination by using the Rose Bengal and *Brucella* Wright tests (both from BioRad, Hercules, CA, USA). Of the 63 patients, 9 had a positive antibody response against a tested antigen (Table): 1 to phase I *C. burnetii* and 8 to *Bartonella* spp. (IgG ≥200). Of these, 7 had a 1-fold dilution higher titer to *B. quintana* than to *B. henselae*, including 1 with a low-level cross-reaction with *C. burnetii*, and 1 with identical titers to both. For all 8 patients, Western blot results were consistent with *Bartonella* endocarditis. For 7, cross-adsorption identified *B. quintana* as the causative species; for the other, the infecting *Bartonella* species remained undetermined because adsorption with *B. quintana* and *B. henselae* antigens removed all antibodies. Serologic results for *B. melitensis* were negative for all patients.

B. quintana is mostly associated with human body lice but has also been found in fleas (*9*). The predisposing factors for *B. quintana* endocarditis are homelessness, alcoholism, and exposure to body lice (*10*). For our patients, the common predisposing factors were poor hygiene and low socioeconomic status, which may expose them to ectoparasites including lice and fleas. In contrast with previous study findings, *B. quintana* infectious endocarditis developed on preexisting valvular lesions in all patients (*10*). This finding may reflect a different clinical evolution than in Europe, where studies have suggested that *B. quintana* infectious endocarditis followed chronic bacteremia in patients who did not have previous valvular defects (*10*).

In summary, prevalence of negative blood culture among patients with infectious endocarditis was high (72%). The most commonly associated risk factor was rheumatic heart disease (Table). *C. burnetii* and *Bartonella* spp. were responsible for 8% of all infectious endocarditis cases and 14% of blood culture–negative cases. No case of infectious endocarditis caused by *B. melitensis* was identified.

Our preliminary study suggests that zoonotic agents, especially *Bartonella* spp., are prevalent causative organisms of blood culture–negative endocarditis in India. We recommend serologic screening for antibodies to zoonotic microorganisms as diagnostic tools for this disease in India.

Nandhakumar Balakrishnan,* Thangam Menon,* Pierre-Edouard Fournier,† and Didier Raoult†

*University of Madras, Taramani, Chennai, India; and †Universite de la Mediterranee, Marseille, France

DOI: 10.3201/eid1407.071374

References

1. Brouqui P, Raoult D. New insight into the diagnosis of fastidious bacterial endocarditis. FEMS Immunol Med Microbiol. 2006;47:1–13. DOI: 10.1111/j.1574-695X.2006.00054.x
2. Koegelenberg CF, Doubell AF, Orth H, Reuter H. Infective endocarditis in the Western Cape Province of South Africa: a three-year prospective study. QJM. 2003;96:217–25. DOI: 10.1093/qjmed/hcg028
3. Benslimani A, Fenollar F, Lepidi H, Raoult D. Bacterial zoonoses and infective endocarditis, Algeria. Emerg Infect Dis. 2005;11:216–24.
4. Tariq M, Alam M, Munir G, Khan MA, Smego RA Jr. Infective endocarditis: a five-year experience at a tertiary care hospital in Pakistan. Int J Infect Dis. 2004;8:163–70. DOI: 10.1016/j.ijid.2004.02.001
5. Li JS, Sexton DJ, Mick N, Nettles R, Fowler VG Jr, Ryan T, et al. Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis. 2000;30:633–8. DOI: 10.1086/313753
6. Dupont HT, Thirion X, Raoult D. Q fever serology: cutoff determination for microimmunofluorescence. Clin Diagn Lab Immunol. 1994;1:189–96.
7. Fournier PE, Mainardi JL, Raoult D. Value of microimmunofluorescence for diagnosis and follow-up of *Bartonella* endocarditis. Clin Diagn Lab Immunol. 2002;9:795–801. DOI: 10.1128/CDLI.9.4.795-801.2002
8. Houpikian P, Raoult D. Western immunoblotting for *Bartonella* endocarditis. Clin Diagn Lab Immunol. 2003;10:95–102. DOI: 10.1128/CDLI.10.1.95-102.2003
9. Marie JL, Fournier PE, Rolain JM, Briolant S, Davoust B, Raoult D. Molecular detection of *Bartonella quintana, B. elizabethae, B. koehlerae, B. doshiae, B. taylorii,* and *Rickettsia felis* in rodent fleas collected in Kabul, Afghanistan. Am J Trop Med Hyg. 2006;74:436–9.
10. 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 (Baltimore). 2001;80:245–51. DOI: 10.1097/00005792-200107000-00003

Address for correspondence: Didier Raoult, Unite des Rickettsies, IFR 48 CNRS, UMR 6020 Universite de la Mediterranee, Faculté de Médecine, 27 blvd Jean Moulin , 13385 Marseille Cedex 05, France; email: didier.raoult@gmail.com

Acute Gastroenteritis Caused by GI/2 Sapovirus, Taiwan, 2007

To the Editor: Sapovirus is an etiologic agent of human gastroenteritis. Although many of the previously reported cases were of mild, sporadic infections in young children (*1–3*), several recent sapovirus-associated gastroenteritis outbreaks have affected adults, which suggests that the virus's virulence, prevalence, or both, may be increasing (*4–6*). In this study, we describe a sapovirus-associated outbreak of gastroenteritis that occurred during May 4–8, 2007, and involved college students in northern Taiwan.

A total of 55 students had clinical symptoms of gastroenteritis, including diarrhea (45), vomiting (22), abdominal cramps (17), and fever (2). The clinical symptoms continued for up to 10 days (mean 4.7 days). Stool

specimens were collected from 8 of 55 students on May 8 (Table). Initially, the specimens were screened for bacteria, rotavirus, and norovirus, but all specimens were negative for these pathogens. The 8 stool specimens were then examined by electron microscopy (EM), and 1 was positive for calicivirus-like particles.

To confirm the EM results, we performed reverse transcription–PCR (RT-PCR), real-time RT-PCR, and sequence analysis as previously described (*7*). Briefly, purified RNA (10 μL) was reverse transcribed by using SuperScript III reverse transcriptase according to the manufacture's instructions (Invitrogen, Carlsbad, CA, USA). PCR was carried out by using the SV-F11 and SV-R1 primer set directed against the conserved N terminal capsid region (*8*). The PCR products were analyzed with 2% agarose gel electrophoresis and visualized after ethidium bromide staining. The PCR-generated amplicons (≈780 bp) were excised from the gel and purified by the QIAquick gel extraction kit (QIAGEN, Hilden, Germany).

Nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1) and determined with the ABI 3130 sequencer (Applied Biosystems, Foster City, CA, USA.). Nucleotide sequences were aligned by using ClustalX (www.clustal.org), and the distances were calculated by using the Kimura 2-parameter method. A phylogenetic tree was generated by the neighbor-joining method as described previously (*1*,*8*).

Of the 8 specimens, 7 were positive by RT-PCR and real-time RT-PCR (Table). SaV124F, SaV1F, SaV5F, and SaV1245R primers as well as SaV124TP and SaV5TP minor-groove binding probes were used for real-time RT-PCR diagnosis, which targets the sapovirus RdRp-capsid junction region as described (*7*). The number of sapovirus cDNA copies ranged from 2.86×10^7 to 1.72×10^{10} copies/g of stool specimen; mean was 2.71×10^9 copies/g of stool specimen (Table). Sequence analysis of the 7 positive specimens showed 100% nucleotide identity (nt 5098–5878), indicating that the outbreak was caused by 1 sapovirus strain.

To better classify the sapovirus, we reamplified the 3′ end of the genome from 1 positive specimen and sequenced ≈2,400 nt (nt 5074-3′) (Hu/SaV/9–5/Taipei/07/TW;GenBank accession no. EU124657). PCR was performed with SV-F13, SV-F14, and TX30SXN primers as described (*1*). Database searches found a closely matching sapovirus sequence (99%) that was detected in a patient with gastroenteritis in Japan, in 2004 (Chiba041413 strain; GenBank accession no. AB258427). The next closely matching sequence was detected in an outbreak of gastroenteritis among adults in the United States in 1994 (Parkville strain; HCU73124) (*6*). Phylogenetic analysis clustered these 3 sapovirus sequences into genogroup I/genotype 2 (GI/2) (online Appendix Figure, available from www.cdc.gov/content/EID/14/7/1169-appG.htm).

Sapovirus was reported in Japan in water samples (untreated wastewater, treated wastewater, and a river) and in clam samples intended for human consumption (*1*). Apart from these 2 environmental studies, little is known about reservoir of sapovirus or its route of infection in the natural environment. The source of contamination in this current outbreak was not determined; however, none of the food handlers associated with the college reported symptoms of gastroenteritis. However, in a recent molecular epidemiologic study in Japan, a large number of symptomatic and asymptomatic food handlers were found to be infected with noroviruses (*9*). Several seroprevalence studies also indicated high prevalence rates of antibodies to sapovirus in adults and children (*10*). All of these findings highlight the need to collect stool specimens from asymptomatic persons and indicate possible "silent" transmission through an asymptomatic route. Symptoms of sapovirus infection are thought to be milder than symptoms of norovirus infections. However, in this study approximately one third (17) of the 55 students reported symptoms of abdominal pain and 22 (40%) reported symptoms of vomiting. Many of the earlier sapovirus studies described sapovirus GI/1 infections in young Japanese children (*1*), which indicated that infecting virus had a different genotype than the virus detected in this study (GI/2).

Table. Clinical symptoms and laboratory diagnosis results for sapovirus-related outbreak among college students, northern Taiwan, May 2007*†

Specimen no.	Patient sex/age, y	Date of illness onset	EM results	RT-PCR results	Copies cDNA/g of stool‡	Symptom			
						Fever	Diarrhea	Vomiting	Abdominal pain
1	F/20	May 5	–	+	1.69×10^8	–	+	+	+
2	F/26	May 5	–	+	6.19×10^8	–	+	+	–
3	M/19	May 6	–	+	2.32×10^8	–	+	–	+
4	M/18	May 6	–	+	3.24×10^8	–	+	+	+
5	F/21	May 7	+	+	1.72×10^{10}	–	+	–	–
6	F/18	May 4	–	–	–	+	+	+	–
7	M/19	May 7	–	+	4.28×10^8	–	+	+	+
8	F/20	May 6	–	+	2.86×10^7	–	+	–	+

*EM, electron microscopy; RT-PCR, reverse transcription–PCR; –, negative; +, positive.
†All specimens were collected May 8.
‡cDNA copies were determined by real-time PCR.

In addition, the viral load in this study appeared to be comparatively high. These results suggest that some sapovirus genotypes are more virulent than others. Similar findings were obtained with norovirus infections around the world; strains belonging to norovirus GII/4 were the most prevalent in many countries. Although several recombinant sapovirus strains have been identified and found to be the cause of increased numbers of infections in some countries (*1*,*5*), they were not observed in this study. Increased sapovirus surveillance and reporting are needed to shed some more light on this poorly understood virus.

This study was supported in part by research grant DOH96-DC-2013 and DOH96-DC-2016 from Centers for Disease Control, Taiwan.

DOI: 10.3201/eid1407.071531

Fang-Tzy Wu,* Tomoichiro Oka,† Naokazu Takeda,† Kazuhiko Katayama,† Grant S. Hansman,† Chih-Hsin Muo,* Shy-Yuan Liang,* Ching-Hsiang Hung,* Donald Dah-Shyong Jiang,* Jui Hsin Chang,* Jyh-Yuan Yang,* Ho-Sheng Wu,* and Chen-Fu Yang*

*Centers for Disease Control, Taipei, Taiwan, Republic of China; and †National Institute of Infectious Diseases, Tokyo, Japan

References

1. Hansman GS, Oka T, Katayama K, Takeda N. Human sapoviruses: genetic diversity, recombination, and classification. Rev Med Virol. 2007;17:133–41. DOI: 10.1002/rmv.533
2. Khamrin P, Maneekarn N, Peerakome S, Tonusin S, Malasao R, Mizuguchi M, et al. Genetic diversity of noroviruses and sapoviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand. J Med Virol. 2007;79:1921–6. DOI: 10.1002/jmv.21004
3. Monica B, Ramani S, Banerjee I, Primrose B, Iturriza-Gomara M, Gallimore CI, et al. Human caliciviruses in symptomatic and asymptomatic infections in children in Vellore, South India. J Med Virol. 2007;79:544–51. DOI: 10.1002/jmv.20862
4. Hansman GS, Saito H, Shibata C, Ishizuka S, Oseto M, Oka T, et al. An outbreak of gastroenteritis due to sapovirus. J Clin Microbiol. 2007;45:1347–9. DOI: 10.1128/JCM.01854-06
5. Hansman GS, Ishida S, Yoshizumi S, Miyoshi M, Ikeda T, Oka T, et al. Recombinant sapovirus gastroenteritis, Japan. Emerg Infect Dis. 2007;13:786–8.
6. Noel JS, Liu BL, Humphrey CD, Rodriguez EM, Lambden PR, Clarke IN, et al. Parkville virus: a novel genetic variant of human calicivirus in the Sapporo virus clade, associated with an outbreak of gastroenteritis in adults. J Med Virol. 1997;52:173–8. DOI: 10.1002/(SICI)096-9071(199706)52:2<173::AID-JMV10>3.0.CO;2-M
7. Oka T, Katayama K, Hansman GS, Kageyama T, Ogawa S, Wu FT, et al. Detection of human sapovirus by real-time reverse transcription-polymerase chain reaction. J Med Virol. 2006;78:1347–53. DOI: 10.1002/jmv.20699
8. Okada M, Shinozaki K, Ogawa T, Kaiho I. Molecular epidemiology and phylogenetic analysis of Sapporo-like viruses. Arch Virol. 2002;147:1445–51. DOI: 10.1007/s00705-002-0821-7
9. Ozawa K, Oka T, Takeda N, Hansman GS. Norovirus infections in symptomatic and asymptomatic food handlers in Japan. J Clin Microbiol. 2007;45:3996–4005. DOI: 10.1128/JCM.01516-07
10. Farkas T, Deng X, Ruiz-Palacios G, Morrow A, Jiang X. Development of an enzyme immunoassay for detection of sapovirus-specific antibodies and its application in a study of seroprevalence in children. J Clin Microbiol. 2006;44:3674–9. DOI: 10.1128/JCM.01087-06

Address for correspondence: Chen-Fu Yang, 161 Kunyang St, Nan Kang, Taipei 115, Taiwan, Republic of China; email: cxy1@cdc.gov.tw

Importation of West Nile Virus Infection from Nicaragua to Spain

To the Editor: We report the case of a 51-year-old Spanish missionary who had lived Nicaragua (Managua) from 2004 to 2006. He had no other notable travel history during that period. In June 2006, he noticed malaise and nausea, followed by abrupt onset of fever (39°C), headache, cervical pain, and right hemiparesis. He was admitted to a local hospital in Nicaragua, at which time routine results of hematologic and biochemistry tests were within normal limits, except for mild neutrophilia. After cerebral magnetic resonance imaging (MRI), a diagnosis of ischemic cerebrovascular accident was made. He was treated with aspirin and ceftriaxone for an oropharyngeal infection.

Because neurologic symptoms persisted, 13 days later he was transferred to a hospital in Madrid, Spain. At that time, physical examination showed neck stiffness, a diminished level of consciousness, right flaccid hemiparesis, and facial weakness. Peripheral blood examination showed only mild neutrophilia. Cerebrospinal fluid (CSF) analysis showed a 65 mg/dL glucose level (blood glucose 140), proteins 136 g/dL, and 18 cells/mm^3 (mainly lymphocytes). Serologic test results for HIV, hepatitis B virus, hepatitis C virus, syphilis, *Toxoplasma* spp., and *Brucella* spp., and CSF cultures for mycobacterial, bacterial, and fungal infections were all negative. Results of a computed tomographic scan of the brain were within normal limits. MRI showed nonspecific abnormal intensity of white matter signal. Electrophysiologic studies showed severe axonal motor neuropathy and moderate sensitive axonal neuropathy in the right upper limb. Gammaglobulin was administered intravenously for 5 days; the patient improved slightly. At

discharge, the diagnosis was of "Guillain-Barré–like syndrome." He was admitted to our Tropical Medicine Unit in Madrid, 160 days after onset of intial symptoms. West Nile virus (WNV) infection was suspected, and diagnostic tests were performed on all available samples. The first serum (S1) and CSF samples obtained 13 days after onset of symptoms were sent to us for testing. A second serum sample (S2) was obtained at 160 days.

The CSF was tested for flavivirus by using a generic PCR (*1*) and found negative, and for WNV immunoglobulin (Ig) G and IgM (Focus, Cypress, CA, USA) as previously described (*2*) and showed positive results for both immunoglobulins (Table). Serum samples were studied by ELISA for WNV IgG and IgM (Focus), and positive results were obtained for IgG to WNV in both samples and for IgM in S1. By a plaque reduction neutralization assay (PRNT) with 100 50% infection units of WNV (Eg-101 strain), positive titers of 256 in S1 and 64 in S2 were obtained (Table).

S1 and CSF were subsequently titrated for WNV IgG by ELISA and for WNV antibodies by PRNT. Specific WNV IgG or total antibodies/albumin indices of 3.54 and 7.0, respectively, were obtained.

Serum samples were also assayed for IgG and IgM by ELISA against dengue virus (Panbio, Brisbane, Queensland, Australia) and tick-borne encephalitis virus (Siemens, Marsburg, Germany). Positive results were obtained for IgG to both viruses; titers did not vary, which suggests cross-reactivity with WNV or prior infection due to another flavivirus.

The causative role of WNV was confirmed by the following factors: 1) the detection of WNV-specific IgM, in the absence of IgM response to the other flavivirus, in combination with the variation of PRNT titer in S1 and S2; 2) the evidence of intrathecal WNV IgG by ELISA and WNV antibodies by PRNT, according to well established cutoff values (*3*); and 3) the detection of WNV-specific IgM in CSF. The final diagnosis was meningoencephalitis with acute flaccid paralysis due to WNV infection with right upper limb paraparesis and muscular atrophy as sequelae (online Appendix Figure, available from www.cdc.gov/EID/content/14/7/1171-appG.htm).

WNV is an arbovirus, family *Flaviviridae,* first detected in 1937. It is maintained worldwide in an enzootic cycle, transmitted primarily between avian hosts and mosquito vectors. Mosquitoes of the genus *Culex* are the main vectors. Humans and horses are accidental secondary hosts (*4*). WNV is now widely distributed in Africa, Asia, the Middle East, Europe, and the Americas. The first epidemics of WNV encephalitis were reported in the early 1950s in Egypt and Israel, then in France (1960s) and in South Africa (1970s). During the past 10 years, several WNV outbreaks in humans have been reported in the Mediterranean basin and in southern Europe (*5*,*6*). In the Americas, the first cases were reported in New York City in 1999 (*7*), and the spread of WNV to large areas of the United States, Canada, Mexico, Central America, and the Caribbean was demonstrated in subsequent years. The case reported here also represents a new case of imported WNV infection in Europe and documents an imported case acquired in Central America (*8–10*)

WNV infection in animals, mainly in birds and horses, has been documented in Mexico, the Caribbean, and areas of South America. Birds, in particular, have been implicated in spreading WNV during migratory events in Europe, Asia, Africa, and the Middle East. WNV could thus potentially be introduced by the same mechanism in Central and South America, resulting in possible transmission to humans in countries like Nicaragua (*7*).

In conclusion, the possibility should be considered of new cases of WNV infection arising outside classic areas of high risk. Clinicians should be aware of the possibility of imported WNV to request specific tests in symptomatic patients.

Support was provided by the Red de Investigación de Centros de Enfermedades Tropicales (RICET).

Begoña Monge Maillo,*
Rogelio López-Vélez,*
Francesca Norman,*
Fernando de Ory,†
María Paz Sanchez-Seco,†
and Cesare Giovanni Fedele†

*Ramon y Cajal Hospital, Madrid, Spain; and †Instituto de Salud Carlos III, Madrid

DOI: 10.3201/eid1407.071496

Table. Serologic results for antibodies to West Nile virus (WNV), dengue virus (DV), and tick-borne encephalitis virus (TBEV)*

Sample/ d after onset	WNV						DV ELISA		TBEV ELISA	
	ELISA				PRNT					
	IgG†	IgG‡	IT-IgG§	IgM†	Ab‡	IT Ab¶	IgG†	IgM†	IgG†	IgM†
S1/13	3.5	65,000	3.54	3.6	256	7.0	4.8	0.3	59.5	0.1
CSF/13		4,000		Pos#	32					
S2/160	3.2	16,000		0.8	64		4.5	0.2	27	0.1

*PRNT, plaque reduction neutralization assay; Ig, immunoglobulin; Ab, antibodies; IT, intrathecal; S1, first serum sample, obtained 13 days after onset of symptoms; CSF, cerebrospinal fluid; Pos, positive; S2, second serum sample, obtained 160 days after onset of symptoms.
†Results expressed as the ratio of sample absorbance to cutoff value (positive >1.0).
‡Titer.
§IT production of WNV IgG (ELISA).
¶IT production of WNV antibodies (PRNT).
#CSF tested diluted 1:2.

References

1. Sánchez-Seco MP, Rosario D, Domingo C, Hernández L, Valdés K, Guzmán MG, et al. Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification. J Virol Methods. 2005;126:101–9. DOI: 10.1016/j.jviromet.2005.01.025
2. Prince HE, Lape-Nixon M, Moore RJ, Hogrefe WR. Utility of the Focus Technologies West Nile virus immunoglobulin M capture enzyme-linked immunosorbent assay for testing cerebrospinal fluid. J Clin Microbiol. 2004;42:12–5. DOI: 10.1128/JCM.42.1.12-15.2004
3. Echevarría JM, Martínez-Martín P, Téllez A, de Ory F, Rapún JL, Bernal A, et al. Aseptic meningitis due to varicella-zoster virus: serum antibody levels and local synthesis of specific IgG, IgM, and IgA. J Infect Dis. 1987;155:959–67.
4. Kramer LD, Li J, Shi PY. West Nile virus. Lancet Neurol. 2007;6:171–81. DOI: 10.1016/S1474-4422(07)70030-3
5. Dauphin G, Zientara S, Zeller H, Murgue B. West Nile virus: worldwide current situation in animals and humans. Comp. Comp Immunol Microbiol Infect Dis. 2004;27:343–55. DOI: 10.1016/j.cimid.2004.03.009
6. Zeller HG, Schuffernecker IBB. West Nile virus: An overview of its spread in Europe and Mediterranean basin in contrast to its spread in the Americas. Eur J Clin Microbiol Infect Dis. 2004;23:147–56. DOI: 10.1007/s10096-003-1085-1
7. Nash D, Mostashari F, Fine A, Miller J, O'Leary D, Murray K, et al. The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med. 2001;344:1807–14. DOI: 10.1056/NEJM200106143442401
8. Charles PE, Zeller H, Bonnotte B, Descasimacker AL, Bour JB, Chavanet P, et al. Imported West Nile virus Infection in Europe. Emerg Infect Dis. 2003;9:750.
9. Gebhardt DO. Another case of West Nile fever in the Netherlands: a man with encephalitis following a trip to Canada. Ned Tijdschr Geneeskd. 2003;147:1336.
10. Hubalek Z, Lukacova L, Halouzka J, Sirucek P, Januska J, Precechtelova J, et al. Import of West Nile virus infection in the Czech Republic. Eur J Epidemiol. 2006;21:323–4. DOI: 10.1007/s10654-006-0019-5

Address for correspondence: Rogelio López-Vélez, Ramón y Cajal Hospital–Infectious Diseases, Carretera de Colmenar 9,1, Madrid 28230, Spain; email: rlopezvelez.hrc@salud.madrid.org

Outbreak of Pertussis, Kabul, Afghanistan

To the Editor: Infectious diseases are the main cause of illness for armed forces in conflict (*1*), resulting in decreases in operational efficiency. The International Security Assistance Force (ISAF) in Afghanistan is a multinational force operating under the auspices of the North Atlantic Treaty Organization (NATO). As part of ISAF, French troops operate in Kabul and its surroundings, within a 70-km radius. French medical facilities consist of a French field hospital and a primary care center. The facilities support 4,000 soldiers, 1,048 of whom are French.

Troop disease, including acute respiratory disease (ARD), is routinely monitored through French Army and NATO surveillance systems. We report an outbreak of ARD in the multinational force in which pertussis cases were identified by using laboratory tests and epidemiologic criteria.

In November 2006, a significant increase of ARD was detected in soldiers of different nationalities (Figure), with a 10-fold increase among French troops at week 51. Patients with persistent cough or dyspnea were referred to the field hospital, in a nonrandomized manner, and those with a 2-week history of cough underwent serologic tests. Samples were sent to France and were analyzed at Hôpital Saint Anne, Toulon, France. Immunoglobulin (Ig) G antibodies to *Bordetella pertussis* antigens (pertussis toxin, filamentous hemagglutinin, and adenylcyclase) were determined by a Western blot assay (MarDx Diagnostics, Carlsbad, CA, USA). Recent infection was diagnosed by finding high levels of antibodies to pertussis toxin compared to results for standardized positive and negative samples, in concurrence with the fact that no soldier had been vaccinated against pertussis after childhood. IgG and IgA antibodies to *Chlamydia pneumoniae* were determined by a semiquantitative method that assessed samples' absorbance value in optical density (SeroCP Quant IgG and Quant IgA, Savyon Diangostics, Ashdod, Israel). Recent infection to *Mycoplasma pneumoniae* was assessed by detecting IgM antibodies with a specific enzyme immunoassay (Platelia *Mycoplasma pneumoniae*, Biorad, Hercules, CA, USA) and by using a semiquantitative method to detect IgM and IgG antibodies with patented gelatin particles sensitized with cell membrane components of *M. pneumoniae* (Serodia Myco II, Fujirebio, Malvern, PA, USA). *Coxiella burnetii* infection was assessed by indirect immunofluorescence assay (*Coxiella burnetii* Spot IF, bioMérieux, Marcy l'Etoile, France).

Statistical analysis was performed with Epi Info v3.4 software package (Centers for Disease Control [CDC], Atlanta, GA, USA). Quantitative variables were compared by using the Kruskall-Wallis test.

From the third week of December 2006 until the third week of January 2007, 209 French soldiers sought treatment at the French medical facilities for stereotyped acute febrile respiratory infection, which represents a cumulative attack rate of 20% on clinical grounds. Thirty-nine French soldiers and 10 non-French soldiers or local civilian workers were then referred to the field hospital. All patients had a 24-h history of fever >38.5°C and nonspecific ear, nose, and throat symptoms, mainly a sore throat. Cough was unremarkable, without whoops. Fourteen of the 49 patients were hospitalized for severe dyspnea. Median age was the same for inpatients (26 [range 20–57] years) and outpatients (36 [range 21–53] years, $p = 0.15$).

Twenty-seven blood samples were taken, 24 from French troops, 2 from British troops, and 1 from Polish patients. Six patients, including 3 French soldiers, had recent pertussis. No difference in age was found between

patients with pertussis and those with non–pertussis ARD (36 [range 27–51] versus 33 [range 20–63] years; p = 0.39). No pertussis patient had been vaccinated against the illness since childhood.

One patient had evidence of recent infection with *M. pneumoniae*, and another with *C. pneumoniae*. No recent infection involved *C. burnetii*. All patients with ARD had a favorable outcome.

This outbreak of ARD among troops in Afghanistan highlights the importance of nontraumatic illness in wartime when military field conditions enhance exposure to, and incidence of, endemic diseases. Although our study did not include systematic laboratory confirmation for all cases of ARD in soldiers due to field conditions, this outbreak was mainly due to pertussis: most cases were defined by a cough lasting ≥2 wk, took place in an outbreak setting, and were (for 6 patients) confirmed by laboratory tests. CDC requirements were followed to ascertain confirmed cases (*2*). This outbreak also involved British troops; after the 2 cases we described, 2 additional serologically confirmed cases and 1 probable confirmed case were discovered among symptomatic British returnees (*3*). Pertussis, which remains endemic in developing countries (*4*), was reported in northeastern Afghanistan in 2002 (*5*), nor was it ever biologically ascertained nor reported in Kabul.

This outbreak elicits 3 main questions. First, how can ARD transmission be stopped under field conditions? Besides prophylactic antibiotherapy, isolation of suspected case-patients is not achievable because of limited number of beds in medical facilities and high-person density in barracks and dining halls. To minimize transmission, patients and caregivers should wear masks.

Second, what prophylactic antibiotherapy should be given? We recommend a 3-day regimen of azithromycin because it is as efficient as erythromycin in preventing spread pertussis (*6*), targets most intracellular bacteria involved in ARD, and offers the best compliance (*7*).

Finally, should soldiers be vaccinated against pertussis for overseas campaigns? In France, no booster vaccination is given after 13 years of age (*8*). Because acellular vaccines do not ensure immunity for >6 years (*5*), no French soldier has immunity to pertussis. We therefore advocate booster vaccination before overseas campaigns. Pertussis vaccination is widely available in combination with vaccination against, at minimum, diphtheria and tetanus, but these combination vaccines can only be performed once in an adult's life and only 2 years after previous vaccination against diphtheria or tetanus. Monovalent vaccines against pertussis must be made more widely available for multinational troops in field conditions.

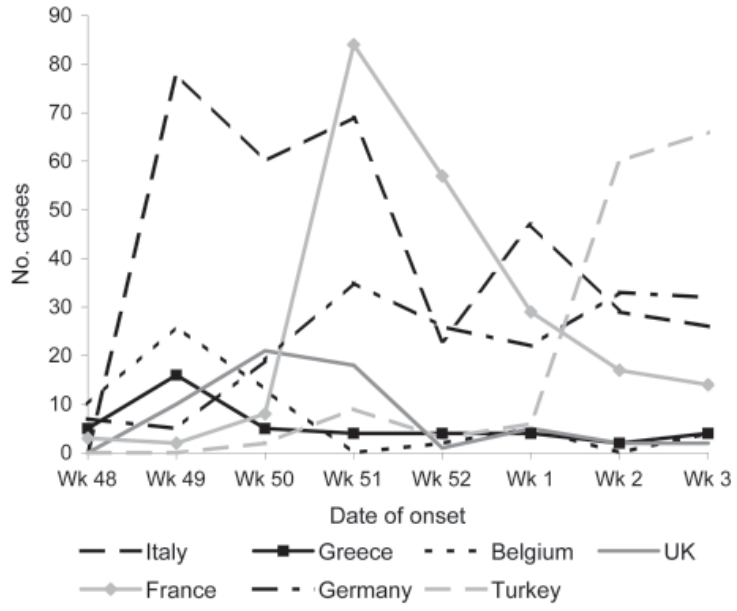


Figure. Number of acute respiratory diseases cases, according to troop nationality. UK, United Kingdom. A color version of this figure is available online: www.cdc.gov/EID/content/14/7/1173-G.htm

Acknowledgments

We are indebted to Martin Powell for helpful comments in preparing the manuscript.

Emmanuel Sagui,*
Lénaïck Ollivier,†
Tiphaine Gaillard‡
Fabrice Simon,*
Patrick Brisou,‡
Philippe Puech,§
and Alain Todesco†

*Hôpital d'Instruction des Armées Laveran, Marseille, France; †Institut de Médecine Tropicale du Service de Santé des Armées, Marseille Armées, France; ‡Hôpital d'Instruction des Armées Saint Anne, Toulon Naval, France; and §Etat Major 4, Marseille Armées

DOI: 10.3201/eid1407.071329

References

1. Sanders JW, Putnam S, Frankart C, Frenck R, Monteville M, Riddle M, et al. Impact of illness and non-combat injury during operations Iraqi Freedom and Enduring Freedom (Afghanistan). Am J Trop Med Hyg. 2005;73:713–9.
2. Centers for Disease Control and Prevention. Case definitions for infectious conditions under public health surveillance. MMWR Recomm Rep. 1997;46:1–55.
3. Cooper NK, Bricknell MCM, Holden GR, McWilliam C. Pertussis—a case finding study amongst returnees from Op Herrick. J R Army Med Corps. 2007;153:114–6.
4. Singh M, Lingappan K. Whooping cough? The current scene. Chest. 2006;130:1547–53. DOI: 10.1378/chest.130.5.1547

5. World Health Organization. Epidemic and pandemic alert and response: pertussis [cited 2007 Aug 5]. Available from http://www.who.int/csr/don/archive/disease/pertussis/en
6. Wirsing von König CH, Halperin S, Riffelmann M, Guiso N. Pertussis of adults and infants. Lancet Infect Dis. 2002;2:744–50. DOI: 10.1016/S1473-3099(02)00452-8
7. Altunaiji S, Kukuruzovic R, Curtis N, Massie J. Antibiotics for whooping cough (pertussis). Cochrane Database Syst Rev. 2005;1:CD004404.
8. Calendrier vaccinal 2006. Bulletin épidémiologique hebdomadaire. 2006;29–30: 212–26.

Address for correspondence: Emmanuel Sagui, Service de Neurologie, Hôpital d'Instruction des Armées Laveran, BP 50, 13013 Marseille, France; email: emmanuel.sagui@laposte.net

Anthropogenic Influence on Prevalence of 2 Amphibian Pathogens

To the Editor: Although the relationship between the emergence of zoonotic diseases and human influenced landscapes is accepted (*1*–*3*), the relationship between human-influenced landscapes and wildlife disease is less so. Evidence does support correlations between human activities and environmental conditions affecting wildlife disease emergence (*2*,*3*). These studies assume relationships between component(s) of human habitat modification and the virulence of disease, and derive estimates of virulence from counts of the visibly diseased or those that have seroconverted (*3*). This explains only part of the host and pathogen dynamic; it seems reasonable to extend the relationship to include prevalence of infection. Data supporting this extension are lacking. Here we present data from a study examining the correlations between human influences on habitat and prevalence of 2 amphibian pathogens (*Batrachochytrium dendrobatidis* and ranavirus FV3) in populations of *Rana clamitans* in central and northeastern Ontario, Canada.

We sampled an average of 25 animals (standard deviation ± 6.16) from 11 populations during summer 2005. We washed equipment in bleach and air-dried equipment between visits and sites. All animals were kept individually to avoid cross-contamination, euthanized with MS22, and assessed for infection using molecular diagnostics. We tested for ranavirus infection of livers by amplifying the major capsid protein using standard PCR (*4*). We tested for infection with *B. dendrobatidis* by using a quantitative real-time PCR (*5*). Prevalence for each pathogen was estimated as the proportion of animals testing positive at a pond.

Site coordinates were determined by using global positioning satellite (GPS), and 4 quantitative measures of human habitat modification were also assessed. GPS coordinates were used to map sites and to measure distance to the nearest road, industrial activity (agriculture, mine, paper mill), and human habitation; all measurements were in meters. We further assigned a qualitative measure of human influence on each breeding pond by assigning ponds to each of the following categories: 1) human presence without human habitat modification or extensive disturbance; 2) recreational activities (fishing, boating); 3) property development (housing or commercial buildings); 4) agricultural activity; and 5) industrial activity. Each of the 5 categories was assigned a 0/1 score; scores for each pond were summed 1–5 (by definition no site scored 0 due to sampling strategy) to derive the final measure of human influence. We modeled the relationship between prevalence and human habitat modification or influence using general linear models (GLM) with prevalence as the dependent variable and with all human influence variables log-transformed to meet assumptions of normality. A type III model structure was used to account for the influence of all explanatory variables in each analysis.

Eight ponds exhibited signs of FV3 infection (range 0%–63% prevalence); 6 ponds contained frogs infected with the amphibian chytrid (range 0%–36% prevalence). GLM did not show any relationship between the prevalence of chytrid infection and all of our explanatory variables (Table). In contrast, 3 of our explanatory variables had a significant influence of ranavirus prevalence. Distance to industrial activity ($p<0.05$), to human habitation ($p<0.05$), and degree of human influence ($p<0.01$) all had a significant effect on the dependent variable (Table).

The disparity between results for the 2 pathogens generates several possible hypotheses. First, proximity to

Table. General linear models for the relationships of amphibian emerging infectious disease prevalence and anthropogenic variables

Data point	Degrees of freedom	Ranavirus		*Batrachochytrium dendrobatidis*	
		Mean squares	F value	Mean squares	F value
Intercept	1	0.06	8.47 ($p<0.05$)	0.05	4.61 ($p<0.1$)
Human disturbance	1	0.24	35.35 ($p<0.01$)	0.0009	0.08
Distance to road	1	0.03	4.11 ($p<0.1$)	0.03	2.82
Distance to industry	1	0.06	8.82 ($p<0.05$)	0.07	5.89 ($p<0.1$)
Distance to housing	1	0.06	8.08 ($p<0.05$)	0.06	4.87 ($p<0.1$)
Error	5	0.01		0.01	

human activities may correlate with the probability of pathogen introduction through introduced species (*6*), fomites, or other sources of infectious particles, with the likelihood of introduction higher for ranavirus. Certainly, both pathogens are presumed to be vectored in association with human activities (*7*,*8*), but *B. dendrobatidis* exhibits a greater host and geographic range and thus should exhibit greater prevalence if humans were mediating introduction across the range of our study. Second, human activities such as construction and industry, may directly or indirectly influence the basic reproductive number, R_0, of ranavirus to a greater extent than for that of *B. dendrobatidis*. Although ranavirus does exhibit optimal environmental ranges for replication and infection, the virulence of *B. dendrobatidis* can be directly influenced by the environment (*2*). Furthermore, infection by *B. dendrobatidis* occurs through a free-living stage; ranavirus is more likely transmitted through direct contact, which suggests that *B. dendrobatidis* would be more sensitive to environmental factors. Last, human activities may influence host ability to mediate immune responses that have the capability to prevent infection. Evidence exists that amphibian host responses to ranavirus are predominantly acquired (*9*); those for *B. dendrobatidis* may be more innate and less prone to environmental manipulation (*10*). Although the observed correlation should be further tested and the disturbance index should be refined, we believe our observed pattern may reflect the influence of human activities and habitat modification in the dispersal of infectious diseases. With increasing evidence pointing towards the role of emerging infectious diseases in the decline of amphibian populations, management plans should therefore account for the indirect effects related to human activities.

Acknowledgments

We thank Albrecht Schulte-Hostedde and Eric Gauthier for use of their molecular equipment for ranaviral analysis. Earlier versions of this manuscript also benefited from comments given by Scott Kaufman, Bruce Pauli, and 2 anonymous reviewers.

This research was funded by NSERC and Laurentian University Research Fund and has been approved by the Laurentian Animal Care Committee (Protocols no. 2005-05-01 and 2005-05-02).

Valerie St-Amour,* Wai M. Wong,† Trenton W.J. Garner,† and David Lesbarrères*

*Laurentian University, Sudbury, Ontario, Canada; and †Institute of Zoology, London, UK

DOI: 10.3201/eid1407.070602

References

1. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. Nature. In press.
2. Bosch J, Carrascal LM, Durán L, Walker S, Fisher MC. Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? Proc Biol Sci. 2007;274:253–60. DOI: 10.1098/rspb.2006.3713
3. Bradley CA, Altizer S. Urbanization and the ecology of wildlife diseases. Trends Ecol Evol. 2007;22:95–102. DOI: 10.1016/j.tree.2006.11.001
4. St.-Amour V, Lesbarrères D. Genetic evidence of *Ranavirus* in toe clips: an alternative to lethal sampling methods. Conserv Genet. 2007;8:1247–50. DOI: 10.1007/s10592-006-9242-6
5. Garner TWJ, Perkins M, Govindarajulu P, Seglie D, Walker SJ, Cunningham AA, et al. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana.* Biol Lett. 2006;2:455–9. DOI: 10.1098/rsbl.2006.0494
6. Garner TWJ, Walker S, Bosch J, Hyatt A, Cunningham A, Fisher MC. Chytrid fungus in Europe. Emerg Infect Dis. 2005;11:1639–41.
7. Jancovich JK, Davidson EW, Parameswaran N, Mao J, Chinchar G, Collins JP, et al. Evidence for emergence of an amphibian iridoviral disease because of human-enhanced spread. Mol Ecol. 2005;14:213–24. DOI: 10.1111/j.1365-294X.2004.02387.x
8. Fisher MC, Garner TWJ. The relationship between the introduction of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. Fungal Biol Rev. 2007;21:2–9. DOI: 10.1016/j.fbr.2007.02.002
9. Robert J, Morales H, Buck W, Cohen N, Marr S, Gantress J. Adaptive immunity and histopathology in frog virus 3-infected *Xenopus.* Virology. 2005;332:667–75. DOI: 10.1016/j.virol.2004.12.012
10. Woodhams DC, Ardipradja K, Alford RA, Marantelli G, Reinert LK, Rollins-Smith LA. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Anim Conserv. 2007;10:409–17. DOI: 10.1111/j.1469-1795.2007.00130.x

Address for correspondence: David Lesbarrères, Biology Department, Laurentian University, Ramsey Lake Rd, Sudbury, Ontario P3B 3C8, Canada; email: dlesbarreres@laurentian.ca

Avian Influenza Virus (H5N1) Mortality Surveillance

To the Editor: The highly pathogenic strain of avian influenza virus subtype H5N1 presents a major challenge to global public health systems. Currently, influenza (H5N1) infection is a zoonosis with a 60% case-fatality rate for affected persons over 3 continents; the virus could mutate to become directly transmissible among humans (*1*). This potential for pandemic transmission must be reduced through early detection of transmission foci, followed by rapid implementation of control measures (*2*). In the following analysis, we demonstrate that single carcasses of birds, mostly found by members of the public, were the primary indica-

tors for avian influenza virus activity in Sweden and Denmark in 2006.

Influenza virus (H5N1) is amplified by commercial and backyard poultry and free-ranging birds. Whether captive birds (e.g., poultry) or wild birds are responsible for the spread of the virus remains a matter of debate (*3*). Initial spread from Southeast Asia before 2005 was likely the result of transport of infected poultry because the spread was not easily explained by natural bird movements (*4*,*5*). However, its spread to Western Europe in late 2005 could be explained by weather-induced migration of waterfowl after a freeze in Eastern Europe (*6*,*7*). Since spreading to Sweden and Denmark in early 2006, the virus has been detected there in dead birds of numerous species (Table). Detections in carcasses of primarily free-ranging birds have become the principal means of tracking spread of the virus in Europe.

To better understand how avian mortality surveillance could be refined for monitoring the spread of influenza virus (H5N1), we analyzed the weekly official reports of such detections in Sweden and Denmark in 2006 (*8*). Virus surveillance in both countries includes both active cloacal swabbing of free-ranging wild birds and passive collection of tracheal swabs from bird carcasses. For the analysis, all carcasses of a single species collected on 1 day within a single locality constituted 1 record. For each record, we evaluated whether the carcasses were reported by a member of the public versus a civil servant, the number of carcasses tested, and the number of positive detections.

Our analysis evaluated 44 records; a total of 70 birds, of 14 species, tested positive for the virus in 22 localities of Sweden and Denmark. Almost all of these records (n≥40, 91%) referred to dead birds found by members of the public rather than civil servants. A smaller portion than expected were Anseriformes (i.e., ducks, geese, or swans; n = 32, 73%). Other orders of birds represented were Falconiformes (hawks, falcons; n = 8, 18%), Strigiformes (owls; n = 2, 5%), Podicepidiformes (grebes; n = 1, 2%), and Charadriiformes (gulls, shorebirds; n = 1, 2%). In addition, birds of other orders tested positive in Denmark but were excluded from the analysis for lack of supporting data. Most (75%) of the records referred to singleton carcasses; the remaining 25% represented multiple detections, ranging from 2 to 9 individual birds of a single species. A majority (73%) of influenza virus (H5N1)–positive localities hosted solely singleton carcasses, whereas the other 27% hosted multiple dead birds. No virus activity was detected through active free-ranging bird surveillance, even though 9,260 live birds were captured and sampled during 2006 in Sweden and Denmark.

The pattern of virus activity observed in Sweden and Denmark was unexpected. Rather than die-offs of large numbers of waterfowl during winter when they congregate, small numbers (mainly singleton birds) were affected late in winter, just before spring migration. During the spring breeding season, less transmission was observed. The predictive power of detecting the virus in free-ranging migratory birds for forecasting poultry outbreaks or human disease remains undetermined. Some of these birds may have been infected in areas remote from the site of detection. However, several of the affected birds in this report were either resident nonmigratory species (eagle owl, Eurasian magpie) or captive domesticated species (muscovy, peafowl, chicken), which indicates local transmission. Health authorities will be better prepared to prospectively minimize transmission in new regions with early warning provided by singleton carcass surveillance.

Surveillance results from Sweden and Denmark highlight the importance of public participation in avian mortality surveillance for influenza virus (H5N1); the preponderance of detections from singleton carcasses; and

Table. Bird species testing positive for highly pathogenic avian influenza virus subtype H5N1, Sweden and Denmark, 2006*

Avian order and species	Scientific name	No. carcasses positive	
		Sweden	Denmark
Podicepidiformes (great crested grebe)	*Podiceps cristatus*	0	1
Anseriformes			
Mute swan	*Cygnus olor*	2	4
Whooper swan	*Cygnus cygnus*	0	3
Greylag goose	*Anser anser*	0	1
Goose spp	*Anser spp.*	1	0
Muscovy duck	*Cairina moschata*	0	2
Mallard	*Anas platyrhynchos*	1	0
Greater scaup	*Aythya marila*	3	0
Tufted duck	*Aythya fuligula*	25	26
Common merganser	*Mergus merganser*	2	0
Smew	*Mergus albellus*	1	0
Falconiformes			
Common buzzard	*Buteo buteo*	1	6
Rough-legged hawk	*Buteo lagopus*	0	1
Peregrine falcon	*Falco peregrinus*	0	1
Galliformes			
Common peafowl	*Pavo cristatus*	0	1
Domestic chicken	*Gallus gallus*	0	1
Charadriiformes (herring gull)	*Larus argentatus*	1	0
Strigiformes (eagle owl)	*Bubo bubo*	2	0
Passeriformes (Eurasian magpie)	*Pica pica*	0	1
All birds		39	48

*Sources: (*6*,*7*).

the broad spectrum of affected species, particularly raptors. A raptor was the index case in Denmark (*7*). Current surveillance efforts in regions free from the virus favor investigation of significant death events of waterfowl and active sampling of healthy waterfowl as the means for early detection (e.g., *9*). Many national surveillance programs are heavily influenced by the influenza virus (H5N1) outbreak in 2005 at Qinghai Lake in China, where hundreds of geese, gulls, and cormorants died during the breeding season (*10*). However, large die-offs may be anomalous or restricted to communal breeding sites of waterfowl where juvenile birds amplify and spread the virus within the breeding colony. Testing of public-reported singleton carcasses provides a more sensitive and robust means of early detection of this virus.

This study was supported in part by the Swedish Research Councils; the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning; and the European Union grant "New Flubird."

Nicholas Komar* and Björn Olsen†

*Centers for Disease Control and Prevention, Fort Collins, Colorado, USA; and †Uppsala University, Uppsala, Sweden

DOI: 10.3201/eid1407.080161

References

1. Peiris JS, de Jong MD, Guan Y. Avian influenza virus (H5N1): a threat to human health. Clin Microbiol Rev. 2007;20:243–67. DOI: 10.1128/CMR.00037-06
2. Mumford E, Bishop J, Hendrickx S, Embarek PB, Perdue M. Avian influenza H5N1: risks at the human–animal interface. Food Nutr Bull. 2007;28:S357–63.
3. Feare CJ. The role of wild birds in the spread of HPAI H5N1. Avian Dis. 2007;51(Suppl):440–7. DOI: 10.1637/7575-040106R1.1
4. Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus AD, Fouchier RA. Global patterns of influenza A virus in wild birds. Science. 2006;312:384–8. DOI: 10.1126/science.1122438
5. Gauthier-Clerc M, Lebarbenchon C, Thomas F. Recent expansion of highly pathogenic avian influenza H5N1: a critical review. Ibis. 2007;149:202–14. DOI: 10.1111/j.1474-919X.2007.00699.x
6. Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP, Daszak P. Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci U S A. 2006;103:19368–73. DOI: 10.1073/pnas.0609227103
7. Bragstad K, Jørgensen PH, Handberg K, Hammer AS, Kabell S, Fomsgaard A, et al. First introduction of highly pathogenic H5N1 avian influenza A viruses in wild and domestic birds in Denmark, Northern Europe. Virol J. 2007;4:43. DOI: 10.1186/1743-422X-4-43
8. Swedish Board of Agriculture. Weekly reports Fågelinfluensa [in Swedish] [cited 2008 June 9]. Available from http://www.sjv.se
9. Cattoli G, Terregino C, Guberti V, De Nardi R, Drago A, Salviato A, et al. Influenza virus surveillance in wild birds in Italy: results of laboratory investigations in 2003–2005. Avian Dis. 2007;51(Suppl):414–6. DOI: 10.1637/7562-033106R.1
10. Liu J, Xiao H, Lei F, Zhu Q, Qin K, Zhang XW, et al. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science. 2005;309:1206. DOI: 10.1126/science.1115273

Address for correspondence: Nicholas Komar, Centers for Disease Control and Prevention, Arbovirus Diseases Branch, 3150 Rampart Rd, Fort Collins, CO 80521, USA; email: nkomar@cdc.gov

Klebsiella pneumoniae Carbapenemase–2, Buenos Aires, Argentina

To the Editor: The activity of carbapenem has been compromised because of the emergence of carbapenemases (*1*). Since 1995, carbapenem resistance has been identified among 77 *Klebsiella pneumoniae* isolates and 1 *Citrobacter freundii* clinical isolate in Argentina (WHONET-Argentina Network). However, until now, none had produced a carbapenemase.

K. pneumoniae carbapenemase-1 (KPC-1) was first detected in a *K. pneumoniae* strain isolated in North Carolina in 2001 (*1*). Since that time, several reports of KPCs worldwide have been made, including in South America (*1*). We report on KPC-2 –producing *K. pneumoniae* and *C. freundii* clinical isolates in Argentina.

A 36-year-old woman with systemic lupus erythematosus and chronic renal failure was admitted to the Sanatorio Mitre in Buenos Aires in September 2006 for a kidney transplant. Two months after the transplant, intraabdominal collection obtained during a surgical procedure yielded a carbapenem-susceptible *Escherichia coli* isolate, after which meropenem therapy was initiated (1 g/day). After 16 days of treatment, an infection developed at the patient's surgical site (per US Centers for Disease Control and Prevention criteria, available from www.cdc.gov/ncidod/dhqp/pdf/guidelines/SSI.pdf). *C. freundii* M9169 and *K. pneumoniae* M9171 were both isolated from the same specimen obtained from the surgical site. Because carbapenemase production was suspected, carbapenem treatment was stopped, and the infection was treated with local antiseptic and drainage for 20 days; the patient was discharged from the hospital in January 2007. Neither the patient nor her relatives or hospital staff had been in the United States before the emergence of these strains.

By using disk diffusion (*2*) (Mueller-Hinton agar and disks obtained from Difco and BBL, respectively; Becton, Dickinson and Co., Franklin Lakes, NJ, USA), we determined that *K. pneumoniae* M9171 was resistant to all antimicrobial drugs except amikacin, tetracycline (*3*), and tigecycline (US Federal Drug Administration criteria, susceptible ≥19 mm). *C. freundii* M9169 remained susceptible

to carbapenems, cefepime, ciprofloxacin, aminoglycosides, chloramphenicol, tetracyclines, and tigecycline but displayed resistance to ceftazidime (CAZ), cefotaxime (CTX), nalidixic acid, and trimethoprim-sulfamethoxazole. When tested with an AmpC-type β-lactamase inhibitor (*4*), both strains showed synergism between 3-aminophenylboronic acid (APB, Sigma-Aldrich, St. Louis, MO, USA) disks and CTX, CAZ, and carbapenems when placed 20 mm apart (center to center). The same synergism was observed for *K. pneumoniae* D5/07, a reference KPC-2–producing strain (College of American Pathologists Quality Control Assurance Program), but not among *E. coli* ATCC 25922. A CMY-2–producing *K. pneumoniae* C2 control strain (*5*) displayed APB synergism against only CTX and CAZ, not carbapenems.

The MICs of carbapenems (*6*) (agar dilution), confirmed disk diffusion results showing a ≥3 doubling-dilution decrease after the addition of APB (300 μg/mL) for *K. pneumoniae* M9171, *C. freundii* M9169, and *K. pneumoniae* D5/07, but not for *K. pneumoniae* C2 and *E. coli* ATCC 25922. Clavulanate (4 μg/mL) reduced only meropenem and ertapenem, and imipenem MICs of *C. freundii* M9169 and *K. pneumoniae* D5/07, respectively (Table).

Isoelectric focusing (IEF) showed that both isolates produced several β-lactamases (Table), including a

Table. Antimicrobial drug susceptibility, isolectric focusing of β-lactamases and PCR of antimicrobial resistance determinants in *Klebsiella pneumoniae*, *Citrobacter freundii* clinical isolates, *Salmonella* transconjugants, and recipient and control strains*

	Clinical isolates		Transconjugants and recipient strains			Control strains		
Type of testing	*K.p.* M9171	*C.f.* M9169	*Salmonella* M9204†	*Salmonella* M9190‡	*Salmonella* M1744	*K.p.* D5/07§	*K.p.* C2¶	*E. coli* ATCC 25922
				MICs (μg/mL)				
Antimicrobial agent								
Imipenem	32	1.0	1.0	1.0	0.12	2.0	0.12	0.25
Impenem/clavulanate	32	0.25	0.25	0.25	0.12	0.5	0.12	0.25
Impenem/APB	2.0	0.12	0.25	0.12	0.12	0.12	0.12	0.25
Imipenem/EDTA#	32	0.25	1.0	1.0	0.12	2.0	0.12	0.12
Meropenem	32	1.0	0.5	1.0	0.015	2.0	0.03	0.03
Meropenem/clavulanate	32	0.12	0.03	0.06	0.015	1.0	0.03	0.03
Meropenem/APB	1.0	0.06	0.03	0.03	0.015	0.12	0.03	0.03
Meropenem/EDTA#	32	1.0	0.5	1.0	0.015	2.0	0.03	0.03
Ertapenem	128	2.0	1.0	1.0	0.008	16	0.06	0.015
Ertapenem/ clavulanate	128	0.25	0.03	0.06	0.008	16	0.06	0.008
Ertapenem/APB	8.0	0.008	0.015	0.03	0.008	0.5	0.03	0.008
Cefoxitin	64	64	4.0	8.0	2.0	ND	ND	4
Ceftazidime	256	16	16	32	0.06	ND	ND	0.25
Ceftazidime/clavulante	16	32	0.5	0.5	0.06	ND	ND	ND
Cefepime	32	4.0	16	16	0.03	ND	ND	0.03
Cefepime/clavulanate	16	0.25	0.5	0.25	0.03	ND	ND	ND
Tigecycline**	1.0	0.25	ND	ND	ND	ND	ND	0.25
				Isolectric focusing results				
pI band††	5.4 + 6.7 + 7.6	5.4 + 6.7 + >9.0	6.7	6.7	None	ND	ND	None
				PCR results				
β-lactamase								
bla$_{KPC}$	+	+	+	+	–	+	–	ND
bla$_{ampC}$	–	+ CIT/ CMY	–	–	–	–	+ CIT/ CMY	ND
bla$_{PER-2}$	+	–	–	–	–	ND	ND	ND
bla$_{SHV}$	+	–	–	–	–	ND	ND	ND
bla$_{TEM-1}$	+	+	–	–	–	ND	ND	ND

**K.p.*, *Klebsiella pneumoniae*; *C.f.*, *Citrobacter freundii*; *E. Coli*, *Escherichia coli*; APB, 3-aminophenylboronic acid; IEF, isoelectric focusing; pI, isoelectric point; ND, not determined; +, positive; –, negative.*
†M9204 transconjugant derived from *K. pneumoniae* M9171.
‡M9190 transconjugant derived from *C. freundii* M9169.
§Control strain producing KPC-2.
¶Control strain producing CMY-2.
#EDTA 0.4 mmol/L.
**Microdilution (JustOne, Trek Diagnostic Systems, Cleveland, OH, USA). Breakpoints according to US Food and Drug Administration (susceptible ≤2 μg/mL).
††Underlined pI bands indicated activity against third-generation cephalosporins.

common enzyme with ESBL activity (isoelectric point [pI] 6.7 by a substrate-based iodometric method) (*7*). *K. pneumoniae* M9171 coproduced another ESBL band at pI 5.4 (*7*).

Beta-lactamases were characterized by PCR specific for KPC (forward primer 5′-AACAAGGAATA TCGTTGATG-3′; reverse primer 5′-AGATGATTTTCAGAGCCTTA-3′), PER-2, SHV, and TEM (*7*). Both strains were PCR-positive for KPC and TEM. PER-2 and SHV were amplified in *K. pneumoniae* M9171. Because of the APB inhibition observed, strains were tested for plasmid-mediated AmpC genes (*8*). The amplicon for the CIT/CMY primers was observed for *C. freundii* M9169 (expectable cross-amplification with chromosomal AmpC) (Table). KPC-type PCR product (916 bp) obtained from *K. pneumoniae* M9171 was sequenced and identified as KPC-2 (*1*).

A wild-type *Salmonella* clinical isolate (M1744) was chosen for conjugational purpose because it naturally lacks AmpCs . Conjugation resulted in the transfer to M1744 of penicillins and third-generation cephalosporin resistance from both clinical isolates (frequency 10^{-4} to 10^{-5}, when selected with ampicillin [50 μg/mL] in *Salmonella–Shigella* medium). Transconjugants showed the acquisition of an ≈70-kb plasmid, which was present in both clinical isolates (*9*). Transconjugants displayed an APB double-disk augmentation trait, further observed by MIC. Only the KPC enzyme was transferred (unique band at pI 6.7) by IEF, which was confirmed by PCR. The absence of plasmid-mediated AmpC genes was confirmed by PCR (Table). No other resistances to non–β-lactam agents were cotransferred.

Carbapenemase activity of crude extracts was measured at 30°C by following 0.4 mmol/L imipenem or ertapenem hydrolysis at 300 nm in 10 mmol/L HEPES (pH 7.5). Addition of 4 mmol/L APB resulted in inhibition of carbapenemase activity of *K. pneumoniae* M9171, *C. freundii* M9169, both transconjugants, and *K. pneumoniae* D5/07, but not a VIM-11 control run in parallel (*10*).

This study identified KPC β-lactamase, which was possessed by 2 strains recovered from 1 patient, in Argentina. Detection of this carbapenemase could become cumbersome because carriage of these genes does not always confer obvious resistance. Moreover, an unusual phenotype was observed in this study; boronic inhibition was associated with the sole presence of KPC. Microbiologists should be aware of cross-reactions (synergism) between APB and KPC that could lead to the false assumption of AmpC-type β-lactamase production, thereby underestimating the presence of this carbapenemase.

Acknowledgments

We are indebted to Martinez-Martinez and Fernandez Canigia for providing *K. pneumoniae* C2 and *K. pneumoniae* D5/07 strains, respectively.

DOI: 10.3201/eid1407.070826

Fernando G. Pasteran,*
Luis Otaegui,*
Leonor Guerriero,*
Gabriel Radice,†
Ricardo Maggiora,†
Melina Rapoport,*
Diego Faccone,* Ana Di Martino,† and Marcelo Galas*

*Instituto Nacional de Enfermedades Infecciosas–ANLIS "Dr. Carlos G. Malbran," Ciudad Autónoma de Buenos Aires, Argentina; and †Sanatorio Trinidad Mitre, Ciudad Autónoma de Buenos Aires

References

1. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. Clin Microbiol Rev. 2007;20:440–58. DOI: 10.1128/CMR.00001-07
2. Clinical and Laboratory Standard Institute. Performance standards for antimicrobial disk susceptibility test: approved standard M2–A9. Wayne (PA): The Institute; 2006.
3. Clinical and Laboratory Standard Institute. Performance standards for antimicrobial disk susceptibility test: seventeenth informational supplement M100–S17. Wayne (PA): The Institute; 2007.
4. Yagi T, Wachino J, Kurokawa H, Suzuki S, Yamane K, Doi Y, et al. Practical methods using boronic acid compounds for identification of class C β-lactamase–producing *Klebsiella pneumoniae* and *Escherichia coli.* J Clin Microbiol. 2005;43:2551–8. DOI: 10.1128/JCM.43.6.2551-2558.2005
5. Pichardo C, Rodríguez-Martínez JM, Pachón-Ibañez ME, Conejo C, Ibáñez-Martínez J, Martínez-Martínez L, et al. Efficacy of cefepime and imipenem in experimental murine pneumonia caused by porin-deficient *Klebsiella pneumoniae* producing CMY–2 β-lactamase. Antimicrob Agents Chemother. 2005;49:3311–6. DOI: 10.1128/AAC.49.8.3311-3316.2005
6. Clinical and Laboratory Standard Institute. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically: performance standards for antimicrobial disk susceptibility test: approved standard M7-A7. Wayne (PA): The Institute; 2006.
7. Melano R, Corso A, Petroni A, Centron D, Orman B, Pereyra A, et al. Multiple antibiotic-resistance mechanisms including a novel combination of extended-spectrum ß-lactamases in a *Klebsiella pneumoniae* clinical strain isolated in Argentina. J Antimicrob Chemother. 2003;52:36–42. DOI: 10.1093/jac/dkg281
8. Pérez-Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol. 2002;40:2153–62. DOI: 10.1128/JCM.40.6.2153-2162.2002
9. Kieser T. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli.* Plasmid. 1984;12:19–36. DOI: 10.1016/0147-619X(84)90063-5
10. Pasteran F, Faccone D, Petroni A, Rapoport M, Galas M, Vázquez M, et al. Novel variant (bla_{VIM-11}) of the metallo-β-lactamase bla_{VIM} family in a GES–1 extended-spectrum β-lactamase–producing *Pseudomonas aeruginosa* clinical isolate in Argentina. Antimicrob Agents Chemother. 2005;49:474–5. DOI: 10.1128/AAC.49.1.474-475.2005

Address for correspondence: Fernando G. Pasteran, Servicio Antimicrobianos, Departamento Bacteriologia, Instituto Nacional de Enfermedades Infecciosas–ANLIS "Dr. Carlos G. Malbrán," Av Velez Sarsfield 563 (1282AFF), Ciudad Autonoma de Buenos Aires, Argentina; email: fpasteran@anlis.gov.ar

Foodborne Diseases

Shabbir Simjee, editor
Humana Press, Totowa, New Jersey, USA, 2007

ISBN-10: 1588295184
ISBN-13: 978-1588295187
Pages: 540; Price: US $149.00

As the title suggests, this book covers relevant topics on foodborne diseases. Most chapters are descriptive, updated, and, given likely page-length limitations, concise. Of 21 chapters, 6 cover selected foodborne bacterial pathogens: *Escherichia coli, Listeria monocytogenes*, *Clostridium botulinum* and *C. perfringens, Yersinia enterocolitica* and *Y. pseudotuberculosis*, pathogenic *Vibrio* spp., and *Enterococcus* spp.

Two chapters discuss viral pathogens such as hepatitis and gastroenteric viruses. Four chapters focus on parasites: *Cryptosporidium* spp., *Cyclospora* spp., *Giardia* spp., and *Toxoplasma gondii*. Other chapters address aflatoxins; scombroid fish poisoning; food management, including hazard analysis and critical control point programs; antimicrobial agents in food-animal production; alternatives to antimicrobial drugs; and microbial risk assessment. Additional chapters also review new trends for control of foodborne pathogens (food irradiation and other sanitation procedures) and molecular techniques for detecting and identifying foodborne pathogens and their toxins. The last chapter considers future directions of food safety.

One limitation of the book is the lack of thorough discussion of other relevant foodborne pathogens, such as *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Bacillus* spp., *Staphylococcus* spp., *Enterobacter sakazakii*, and *Aeromonas* spp. These bacteria are important foodborne pathogens worldwide, and although they are briefly mentioned in other chapters, much more consideration is warranted (*1*,*2*).

For example, in 2006, in the United States, 5,712 cases of *Campylobacter* infection and 6,655 cases of *Salmonella* infection were documented. *Campylobacter* spp. are the most frequently diagnosed causes of gastroenteritis in the United States, and ≈80% of cases are foodborne. Recent well-publicized foodborne outbreaks in the United States have been associated not only with *E. coli* O157:H7, but with *Salmonella* spp. as well. A multistate outbreak of *S.* Typhimurium infections associated with tomatoes accounted for 14% of the cases in 2006. *S.* Newport accounted for 9.2% of the cases. In 2007, >400 cases of *S.* Tennessee infection were attributed to consumption of peanut butter. *E. sakazakii* has caused fatal infections in neonates who were fed contaminated infant formula; this pathogen presents particular challenges to the food industry. In addition, no mention was made of helmintic infections, which also can be associated with foods.

If page limitations were an issue, the 4 chapters dedicated to parasitic infections could have been condensed to 2, and bacterial pathogens could have been emphasized. This would have been doable because 3 of the 4 parasite chapters were written by the same senior author in collaboration with others.

Overall, several relevant topics on foodborne diseases are sufficiently described in this book, and credit should be given to the chapter contributors who provided adequate information on their respective topics. This is a very good reference book for health departments, the food industry, and academia.

Ynes R. Ortega*

*University of Georgia, Griffin, Georgia, USA

DOI: 10.3201/eid1407.080346

References

1. Centers for Disease Control and Prevention. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food—10 states, 2006. MMWR Morb Mortal Wkly Rep. 2007;56:336–9.
2. Altekruse SF, Stern NJ, Fields PI, Swerdlow DL. *Campylobacter jejuni*—an emerging foodborne pathogen. Emerg Infect Dis. 1999;5:28–35.

Address for correspondence: Ynes R. Ortega, University of Georgia, Center for Food Safety, 11098 Experiment St, Griffin, GA 30223, USA; email: ortega@uga.edu

Husband

Connie Smith Watson

She always brushed her hair back

Sternly, twisting one small knot about the crown.

He did not like the plain, broad brow.

"I hate that tightness," he would mutter to himself.

But sometimes he would stoop to kiss her forehead

And at midnight, hesitatingly, her lips,

Surprised each time the way her arms would reach for him.

When little Thomas had diphtheria at two,

Her back was hourly bent above the crib,

And one day as he stood beside her

He too bent, drawn helplessly, and

Kissed the tender whiteness of her neck,

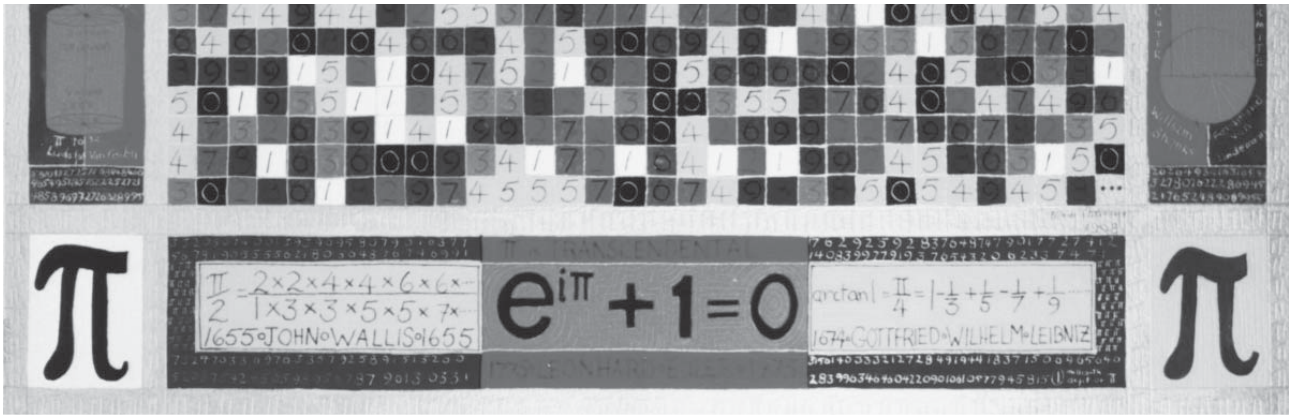
The unruly tendrils capturing his lips.

With frost-cracked palm he stroked

Her disciplined small head,

And never thought to mutter any more.

Mrs. Watson has been writing poetry and prose for more than 70 years. Her poetry has won numerous awards, including the Editor's Choice Award from the Georgia State Poetry Society, and has been featured in publications including Home Life, Leatherneck, and Writer's Digest. She resides in Fitzgerald, Georgia.



Anne Adams (1940–2007). *Pi* (1998). Gouache on paper. Used with permission of Robert A. Adams.

"Much Madness is Divinest Sense"

—Emily Dickinson

Polyxeni Potter*

"And yet I still had so much music in my head," lamented Maurice Ravel (1875–1937) near the end of his life (*1*). The French composer was frustrated by symptoms of an undiagnosed neurologic disorder that interfered with his ability to move, speak, or express creative ideas. Now labeled primary progressive aphasia-related illness, the disorder also marked the life and art of Anne Adams.

A native of Canada, Adams was educated in the sciences and excelled in physics and chemistry, which she taught at the college level. During an interval from academe, she raised her four children then returned to the sciences at age 35 as a student of cell biology, professor, and researcher. At age 46, she left again, this time to nurse her son who had been injured in an automobile wreck. The injury resolved much faster than anyone expected, but Adams decided not to return to science but pursue other interests. A lover of music and the arts, she had dabbled with painting in her earlier years, mostly architectural drawing and watercolor in a classical style.

Over the next few years, she became increasingly absorbed with art, devoting all her days to work in her studio. Her style and technique evolved rapidly, and she started experimenting, particularly with expression of sounds as visual forms. She interpreted musical scores and converted them to colorful images (Rondo alla Turquoise, Rhapsody in Blue). She became fascinated with the music of Maurice Ravel, particularly his one-movement orchestral piece Boléro.

"Don't you think this theme has an insistent quality?" Ravel asked his friend Gustave Samazeuilh as he fingered the initial melody on the piano, "I'm going to try and repeat it a number of times without any development, gradually increasing the orchestra as best I can" (*2*). This he did. Two melodic themes were repeated eight times over 340 bars. Volume and instrumentation increased along with two alternating staccato bass lines. There was no key change until the 326th bar, when the piece accelerated into a collapsing finale (*3*). The result was haunting and infectious, an exercise in compulsion, some said perseveration.

Ravel wrote this his best-known composition while on vacation in the south of France. He was 53. Though the musical scores were marred with spelling errors, he was not yet incapacitated by illness. The success of Boléro, which he had assessed as "a piece for orchestra without music," surprised him. During the premier of the work, a woman was said to exclaim that the composer was mad. Ravel later remarked that she must have understood the piece (*4*).

Anne Adams knew nothing of Ravel's illness or her own. But at age 53, she started to work on the painting Unraveling Boléro, a visual analysis of Ravel's composition.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: 10.3201/eid1407.000000

She transformed the music into colorful figures, one for each bar. Highly structured and rendered with meticulous detail, they resembled spiky space-age lace hung out to dry in neat monotonous rows. The height of figures corresponded with volume, the shape with note quality, the color with pitch.

In *Pi*, on this month's cover of Emerging Infectious Diseases, painted when Adams was 58 and before any symptoms of language loss, she moved away from translating music toward abstraction. At the peak of her creativity, she painted mathematical concepts. And it is not surprising that she chose to paint π, one of the most mysterious and recognizable numbers, even to those who have long forgotten what it represents or how frequently it turns up in science and nature. Inside an iconic border summarizing the history of π, Adams portrayed a 32- × 46-digit portion in a matrix of the first 1,471 digits (plus the decimal point). With white, black, and component colors of the white light spectrum marking each integer from 0 to 9, she tried to capture the randomness of π's expansion.

Loss of language (difficulty with grammar, syntax, articulation, speech) and motor function (declining muscle control), main symptoms of Adams' (and Ravel's) illness, have long been known to neurologists as the result of lesions on the left frontal lobe. What was extraordinary in these two cases was the simultaneous increase in capabilities of the posterior right region of the brain. Ravel died at 62 of complications after neurosurgical treatment, Adams at 67 of aspiration pneumonia brought on by severe motor and respiratory decline.

Neuropathy, with its dreaded sequelae, is a common prospect for an aging population, and not only as it relates to primary progressive aphasia. Meningitis, the scourge of children and youth as well as the immunocompromised, has multiple infectious causes and disastrous outcomes when left undiagnosed and untreated. The epidemiology of bacterial meningitis around the world keeps evolving, impeding vaccine development (*5*). Illness caused by emerging pathogens (e.g., *Rickettsia felis*) is likely underreported (*6*). Meanwhile interspecies hybrids of pathogenic yeasts that can cause meningoencephalitis (e.g., *Cryptococcus neoformans* and *C. gattii*) are now found in patients with weakened immune systems (*7*).

Unlike Adams and Ravel, most patients with neurologic disorders experience no unusual creative powers. They face a degenerative clinical course and early death. But the spark of genius, even when ignited by illness, may shed light on unexplored areas of the mind, although how the brain supports the creative process remains as much a mystery as π.

Mathematicians and artists alike have turned to repetition and exquisite detail in their search for perfection. And so have public health researchers. Exhaustive reporting and integration of surveillance data can identify specimens for genetic analysis and clarify variants associated with susceptibility to central nervous system disease (*8*).

References

1. Jourdan-Morhange H. Ravel et nous. Genève: Ed. du Milieu du Monde; 1945.
2. Orenstein A. The ballets of Maurice Ravel: creation and interpretation. Burlington (VT): Ashgate; 1991.
3. Seeley WW, Matthews BR, Crawford RK, Gorno-Tempini ML, Foti D, Mackenzie IR, et al. Unravelling Boléro: progressive aphasia, transmodal creativity and the right posterior neocortex. Brain. 2008;131:39–49. DOI: 10.1093/brain/awm270
4. Kavanaugh P. Music of the great composers. Grand Rapids (MI): Zondervan; 1996.
5. Ceyhan M, Yildirim I, Balmer P, Borrow R, Dikici B, Turgut M, et al. A prospective study of etiology of childhood acute bacterial meningitis, Turkey. Emerg Infect Dis. 2008;14:1089–96.
6. Pérez-Osorio CE, Zavala-Velázquez JE, León JJA, Zavala-Castro JE. *Rickettsia felis* as emergent global threat for humans. Emerg Infect Dis. 2008;14:1019–23.
7. Bovers M, Hagen F, Kuramae EE, Hoogveld HL, Dromer F, St-Germain G, et al. AIDS patient death caused by novel *Cryptococcus neoformans* × *C. gattii* hybrid. Emerg Infect Dis. 2008;14:1105–8.
8. Crawford DC, Zimmer SM, Morin CA, Messonnier NE, Lynfield R, Yi Q, et al. Integrating host genomics with surveillance for invasive bacterial diseases. Emerg Infect Dis. 2008;14:1138–40.

Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; email: PMP1@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Bacterial Pneumonia and Pandemic Influenza Planning

Deaths from Bacterial Pneumonia during 1918–19 Influenza Pandemic

Community Strains of MRSA and Healthcare-Associated Infections, Uruguay, 2002–2004

Zoonotic Transmission of Simian Foamy Viruses in Asia

Puumala Hantavirus Excretion Kinetics in Bank Voles

Rift Valley Fever Virus Seropositivity, Northeastern Kenya

Systematic Literature Review of Role of Human Noroviruses in Sporadic Gastroenteritis

Zika Virus in Yap State, Micronesia, 2007

Study of Buruli Ulcer Disease and Potential Aquatic Invertebrate Vectors

Genotyping *Rickettsia prowazekii* Isolates

Infection with Panton-Valentine Leukocidin–Positive MRSA t034

Cutaneous Infrared Thermometry for Detecting Febrile Patients

Environmental Contamination during Influenza Virus (H5N1) Outbreaks, Cambodia, 2006

Identification of Residual Blood Proteins in Ticks by Proteomics

Increased Amoxicillin–Clavulanic Acid Resistance in *Escherichia coli* Blood Isolates

Detection and Molecular Characterization of a Canine Norovirus

Isolation and Molecular Characterization of Banna Virus from Mosquitoes

Oseltamivir Prescribing, United States, 2004–2005

Cluster of Falciparum Malaria Cases in UK Airport

Circulation of European Bat Lyssavirus Type 1 in Serotine Bats

Henipavirus Infection in Fruit Bats (*Pteropus giganteus*), India

Hemolytic Uremic Syndrome–associated Enterohemorrhagic *Escherichia coli*

Macrolide-Resistant *Shigella sonnei*

Complete list of articles in the August issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 5 –15, 2008
IUMS 2008
Meetings of the Three Divisions of the International Union of Microbiological Societies
Istanbul, Turkey
http://www.iums2008.org/

October 30–November 1, 2008
Ninth International Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases Meeting (MEEGID IX)
University of California at Irvine
Irvine, CA, USA
http://www.th.ird.fr/site_meegid/menu.htm

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

The Public Health Image Library (PHIL)

The Public Health Image Library (PHIL), Centers for Disease Control and Prevention, contains thousands of public health-related images, including high-resolution (print quality) photographs, illustrations, and videos.

PHIL collections illustrate current events and articles, supply visual content for health promotion brochures, document the effects of disease, and enhance instructional media.

PHIL Images, accessible to PC and Macintosh users, are in the public domain and available without charge.

Visit PHIL at http://phil.cdc.gov/phil

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscape.com/cme/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Alcaligenes xylosoxidans Bloodstream Infections in Outpatient Oncology Office

CME Questions

1. Which of the following statements about *Alcaligenes xylosoxidans* is most accurate?

A. *A. xylosoxidans* is gram-positive
B. *A. xylosoxidans* is hydrophobic
C. *A. xylosoxidans* is associated with healthcare-associated infections
D. *A. xylosoxidans* is found only in soil

2. Which of the following statements about the clinical presentation of *A. xylosoxidans* infection in the current study is most accurate?

A. No patients with *A. xylosoxidans* infection were immunocompromised
B. Patients with *A. xylosoxidans* were homogeneous with regard to age and clinical diagnosis
C. Patients with *A. xylosoxidans* infection had symptoms that were attributed to side effects of chemotherapy
D. Only 10% of patients with *A. xylosoxidans* infection were successfully treated with antibiotics

3. Which of the following factors was most different in comparing case-patients and control groups in the current study?

A. Case-patients with *A. xylosoxidans* infection had a lower mean white blood cell count compared with controls
B. Case-patients with *A. xylosoxidans* infection were more likely to have a central venous catheter compared with controls
C. Case-patients with *A. xylosoxidans* infection had a greater number of intravenous medications administered compared with controls
D. Case-patients with *A. xylosoxidans* infection had more underlying diseases compared with controls

4. Which of the following sources was most likely the primary reservoir of *A. xylosoxidans* in the current study?

A. Tap water
B. Mixing hood
C. Hospital personnel
D. Multidose vials of heparin and saline flushes

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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 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 (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, 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. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

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. 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. 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. 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 a 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. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.