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September 2009

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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.

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On the Cover

Abu'l Hasan, Nadir al-Zaman (1588–c.1635)
Squirrels in a Plane Tree with Hunter attempting to Climb the Tree (1605–08)
Gouache on paper (22 cm × 36 cm)
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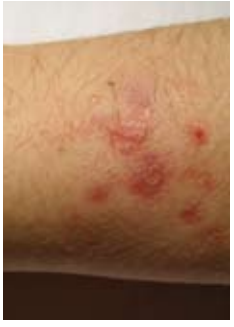

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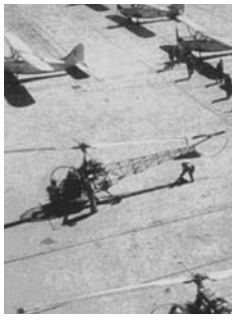

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Using Satellite Images of Environmental Changes to Predict Infectious Disease Outbreaks

Timothy E. Ford, Rita R. Colwell, Joan B. Rose, Stephen S. Morse, David J. Rogers, and Terry L. Yates[1]

Recent events clearly illustrate a continued vulnerability of large populations to infectious diseases, which is related to our changing human-constructed and natural environments. A single person with multidrug-resistant tuberculosis in 2007 provided a wake-up call to the United States and global public health infrastructure, as the health professionals and the public realized that today's ease of airline travel can potentially expose hundreds of persons to an untreatable disease associated with an infectious agent. Ease of travel, population increase, population displacement, pollution, agricultural activity, changing socioeconomic structures, and international conflicts worldwide have each contributed to infectious disease events. Today, however, nothing is larger in scale, has more potential for long-term effects, and is more uncertain than the effects of climate change on infectious disease outbreaks, epidemics, and pandemics. We discuss advances in our ability to predict these events and, in particular, the critical role that satellite imaging could play in mounting an effective response.

Atmospheric chemists and climate modelers have little doubt that the earth's climate is changing. Concomitant with rising carbon dioxide levels and temperatures, severe weather events are increasing, which can lead to substantial rises in sea level, flooding, increased droughts, and forest fires (*1*). In recent decades, infectious diseases have resurged, and previously unrecognized agents of disease have been characterized (*2*). Evidence is accruing that these phenomena may in part be linked to environmental change (*3*). Several questions have emerged from events that have occurred over the past 20 years: was cryptosporidiosis inevitable in Milwaukee, Wisconsin, USA, in 1993, and was *Escherichia coli* O157 infection inevitable in Walkerton, Ontario, Canada, in 2000? Both events were preceded by heavy rains; had highly concentrated sources of pathogens in the form of untreated sewage and animal waste, respectively; and had vulnerable infrastructure. Although the situations were perhaps more complex, could we have predicted epidemic cholera in South America in 1991 after a 100-year absence and the emergence of a new strain of potentially pandemic cholera in India in 1992?

A considerable body of knowledge has accumulated over the past decade or so about the relationships between environment and disease, yet far more information and resources are needed if we are to develop effective early warning systems through environmental surveillance and modeling as well as appropriate emergency response. In the United States, we face a crisis in funding that not only affects basic and applied research in this field but also undermines our ability to deploy remote sensing technologies that provide the most promising means for monitoring our environment. Using examples of waterborne and vectorborne disease, we will discuss how remote sensing technology can be used for disease prediction. We will then examine the lessons learned from these examples and provide recommendations for future modeling.

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DOI: 10.3201/eid1509.081334

[1]Deceased.

Waterborne Disease

Water and climate go hand in hand, with precipitation and extreme events known to be associated with waterborne outbreaks (*4*). Flooding is the most frequent natural weather disaster (30%–46% of natural disasters in 2004–2005), affecting >70 million persons worldwide each year (data for 2005 [*5*]).

The most common illnesses associated with floods described in the literature are diarrhea, cholera, typhoid, hepatitis (jaundice), and leptospirosis. Unusual illnesses such as tetanus have also been reported. The etiologic agents identified include *Cryptosporidium* spp., hepatitis A virus, hepatitis E virus, *Leptospira* spp., *Salmonella* spp., and *Vibrio* spp. Severe outbreaks of cholera, in particular, have been directly associated with flooding in Africa and in West Bengal, India (*6*,*7*).

A rise in sea level, combined with increasingly severe weather events, is likely to make flooding events commonplace worldwide. The Climate Change 2001 Synthesis Report from the Intergovernmental Panel on Climate Change (*8*) suggests that the average annual numbers of persons affected by coastal storm surges will increase from <50 million at present sea levels to ≈250 million by the 2080s, assuming a 40-cm rise in sea level. Even with enhanced protection through engineering interventions, this number is anticipated to reach ≈100 million persons. The initial proportion of deaths from these events is huge, but without extreme vigilance and better monitoring and response, major epidemic waterborne diseases will continue to occur. Factors that promote waterborne disease—overcrowding, lack of sanitation, lack of clean water, certain domestic animal practices, waste disposal—are exacerbated by flooding.

Using Satellite Technology to Model Prediction of Cholera Outbreaks

Effective prediction depends on many factors, not just the prediction of an event. Cholera may be the most studied and best understood of the waterborne diseases and, perhaps in hindsight, we could have predicted the occurrence of cholera in South America in 1991 (*9*). Models for cholera prediction, although country specific, are constantly improving. For example, considerable work has gone into predicting outbreaks of cholera in Bangladesh. Remote imaging technologies developed by the US National Aeronautics and Space Administration have been used to relate sea surface temperature, sea surface height, and chlorophyll A levels to cholera outbreaks (Figure 1) (R.R. Colwell and J. Calkins, unpub. data). This process used a composite environmental model that demonstrated a remarkable similarity between predicted rates based on these 3 parameters and actual cholera incidence. These data are far from perfect and considerable uncertainty still remains. For example, rates of cholera were much higher than predicted in January 1998 and January 1999, yet many of the predicted peaks closely aligned with actual incidence. Because the model is constantly being improved and the satellite data are becoming increasingly accurate through ground truthing (real-time collection of information on location), we believe that satellite imaging provides tremendous promise for prediction of cholera, weeks and even months in advance of an epidemic.

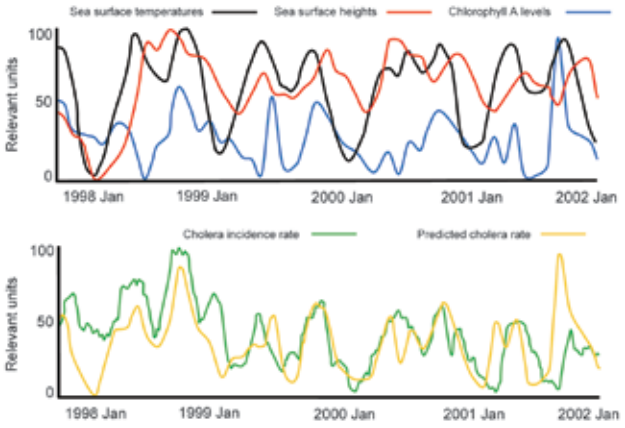

Figure 1. Modeling cholera outbreaks in Bangladesh. Adapted from R.R. Colwell and J. Calkins, unpub. data.

Knowing when an outbreak is likely to occur can inform public health workers to stress basic hygiene and sanitation and to implement simple mitigation efforts such as filtration of water with sari cloth, which in some areas is credited with reducing deaths from cholera by >50% (*10*). Although remote sensing technology is currently still a research tool, the example of cholera prediction through its use provides a compelling argument to maintain and adequately fund our satellite programs; unless this is done, this extraordinary effort at disease prediction will fail.

Some of the critical needs that must be met to predict the effect of environmental change on waterborne disease include the following: 1) better knowledge of disease incidence and pathogen excretion; 2) better characterization of the pathogens in sources (e.g., combined sewer overflows, septic tanks) and these sources' vulnerabilities to climate change; 3) better monitoring of sewage indicators to gather source, transport, and exposure information (event monitoring); 4) improved understanding of sediments and other pathogen reservoirs; 5) more quantitative data for risk assessment; and 6) better health surveillance data. In turn, this information can be used to better use ground truthing in combination with remote sensing technologies as predictors of waterborne disease outbreaks.

Vector-borne Disease

Other emerging and reemerging infectious diseases also are environmentally driven. Many are zoonotic, vector-

borne, or both, and have complex life histories that make predicting disease emergence or reemergence particularly difficult. An insect or rodent vector can make it almost inevitable that a pathogen will be globally transported by plane or boat. With environmental change, disease range, prevalence, and seasonality may change in direct relationship to the vector or animal host. Therefore, to understand the life cycle of a pathogen and the risks of disease emergence, all stages of that life cycle and the life cycles of its intermediate hosts must be considered.

To date, predicting vector-borne diseases has proved to be complex. Although climate change and other environmental stressors are major components, separation from human factors is difficult. Climate change undoubtedly affects the distribution of disease, but changes in human behavior that increase exposure risk are also critical factors. Šumilo et al. (*11*) reported that climatic variables explain only 55% of spatial variation in tick-borne encephalitis in the Baltic States, which have seen an increase in disease incidence over the past 3 decades. These authors report that changes in predation pressure on intermediate hosts and shifting socioeconomic conditions that increase or decrease peoples' visits to forests (for recreation, work, or berry and mushroom harvesting) are important factors in disease distribution (*12*).

Effective modeling of future risk for vector-borne disease outbreaks needs to take into account human behavior that increases exposure, as well as other factors that effect the ecology of the vectors, such as predation pressure and habitat change. Coupled with remote sensing technologies that monitor environmental and climatic changes, human observations of population movement and distribution will be necessary.

Malaria also presents a challenge. This disease continues to devastate sub-Saharan Africa and other parts of the developing world. Substantial resources over the past several decades have gone toward eradication, vaccination, treatment, and, more recently, prediction of malaria outbreaks. Satellite imaging has been used to predict the distribution of 5 of the 6 *Anopheles gambiae* complex species that are responsible for much of the malaria transmission in Africa (*13*). However, human factors again make accurate prediction of disease events complex. Prediction of a disease event is complicated by host immunity effects, which can result in cycles of infection that would appear to bear no relationship to environmental variables. To predict malaria outbreaks, remote sensing technologies need to be coupled with a better understanding of how specific populations are effected by host immunity, which could allow population susceptibility at any given time to be estimated.

Using Satellite Technology to Model Hantavirus Pulmonary Syndrome

Although considerable uncertainty exists in disease prediction through remote sensing technology, particularly for vector-borne disease as discussed above, satellite technology has been applied with some success to predictive modeling for cases of hantavirus pulmonary syndrome (HPS). The 1993 outbreak of HPS in the southwestern United States was believed to be linked to environmental conditions and, in particular, to abnormally high rainfall that resulted in increased vegetation with a subsequent explosion in the rodent populations. Several research groups have subsequently modeled conditions that led to an HPS outbreak, with mixed success. Engelthaler et al. (*14*) looked at 10 years of data on monthly precipitation and daily ambient temperature in the Southwest region (1986–1995) in relation to HPS cases (1993–1995). They found that cases tended to cluster seasonally and temporally by biome type and elevation and only indirectly demonstrated a possible association between the 1992/1993 El Niño precipitation events and HPS. Glass et al. (*15*,*16*) were also unable to make a definitive link with precipitation events in their analyses of HPS in the southwestern United States. They did, however, find a relationship between Landsat Thermatic Mapper (LTM) images recorded by satellite in 1992 and HPS risk the following year. LTM generates numbers that represent reflected light in 6 bands, 2 of which were associated with decreased risk and 1, in the mid-infrared range, with increased risk. The authors admit that considerable ground truthing is necessary to relate satellite imagery to the environmental variables being measured (i.e., vegetation, soil type, soil moisture) and their relation to rodent population dynamics.

However, this work does demonstrate the utility of remote satellite imaging and the increasingly important role it can and should play in disease prediction. In 2006, Glass et al. (*17*) reported strong predictive strength from logistic regression modeling of LTM imagery from 1 year, when estimating risk of HPS the following year, for the years 1992–2005. Their risk analysis for 2006, based on Landsat imagery for 2005, when precipitation levels increased dramatically over prior drought years, suggested an increased risk for HPS, particularly in northern New Mexico and southern Colorado. This prediction was unfortunately borne out in the early part of 2006 when 9 cases of HPS occurred within the first 3 months, 6 of those cases in New Mexico and Arizona. However, the anticipated threat to Colorado did not occur, with a fairly typical number of 6 cases, compared with a total of 11 cases for the state in 2005 (*18*).

However, these results are not necessarily a failure of prediction. In fact, they may illustrate that an early warning system serves to reduce exposure of persons to the deer

mice habitat. For example, USA Today highlighted HPS risks with a June 8, 2006, article titled "Officials warn of increased threat of hantavirus" (www.usatoday.com/news/health/2006-06-08-hantavirus-x.htm). The role of the popular press is hard to quantify but undoubtedly does have an effect on human behavior patterns. Many health departments in the western states produce health advisories warning the public about the risks of exposure to the virus through inhalation of dust contaminated with rodent urine, feces, or saliva. The popular press may serve an important role in increasing awareness of a heightened health risk, which, in turn, promotes greater compliance with health advisories.

Lessons Learned and Recommendations for Future Modeling

The scientific community has a relative consensus that epidemic and pandemic disease risks will be exacerbated by environmental changes that destabilize weather patterns, change distribution of vectors, and increase transport and transmission risk. Predictive modeling may lead to improved understanding and potentially prevent future epidemic and pandemic disease. Many respiratory infections are well known as highly climate dependent or seasonal. Although we are not yet able to predict their incidence with great precision, we may well be able to do this in the future. Meningococcal meningitis (caused by *Neisseria meningitidis*) in Africa is probably the best known example. In the disease-endemic so-called meningitis belt (an area running across sub-Saharan Africa from Senegal to Ethiopia), this is classically a dry season disease, which ceases with the beginning of the rainy season, likely as a result of changes in host susceptibility (*19*). Many other infectious diseases show strong seasonality or association with climatic conditions (*20*). Perhaps one of the most interesting is influenza, which is thought of as a wintertime disease in temperate climates but shows both winter and summer peaks in subtropical and tropical regions (*21*). Although the reasons for seasonality are often poorly understood, the close dependence of such diseases on climatic conditions suggests that these, too, are likely to be amenable to prediction by modeling and remote sensing (*22*).

When we consider influenza, it is hard not to think about the future risks from pandemic influenza. Public health agencies in the United States and around the world are focusing on influenza preparedness, notably concerning influenza virus A subtype H5N1, which has captured attention because it causes severe disease and death in humans but as yet has demonstrated only very limited and inefficient human-to-human transmission. The severity of the disease raises images of the 1918 influenza epidemic on an unimaginably vast scale if the virus were to adapt to more efficient human-to-human transmission. Can predictive modeling using satellite or other imaging of environmental variables help in prediction of future influenza pandemics? Xiangming Xiao at the University of New Hampshire was funded in 2006 by the National Institutes for Health to lead a multidisciplinary and multi-institutional team to use remote satellite imaging to track avian flu. Xiao et al. have used satellite image–derived vegetation indices to map paddy rice agriculture in southern Asia (*23*). They believe that a similar approach can be used in conjunction with the more traditional approach of analyzing bird migration patterns and poultry production (*24*,*25*) to map potential hot spots of virus transmission (*26*).

An interesting question is why did we not see disease epidemics in Indonesia, following the devastating tsunami disaster of December 2004? Could rapid public health intervention be credited with minimizing spread of disease? In the case of Aceh Province, many communities reported diarrhea as the main cause of illness (in 85% of children <5 years of age), but no increases in deaths were reported, and no outbreaks of cholera or other potentially epidemic diseases occurred (*27*). Given the massive scale of the disaster, was this likely? In some towns, more than two thirds of the population died at the time of impact, almost 100% of homes were destroyed, and 100% of the population lacked access to clean water and sanitation (*27*). To a large extent, the Australian army and other groups are to be credited with rapidly deploying environmental health teams to swiftly implement public health measures, including provision of safe drinking water, proper sanitary facilities, and mosquito control measures (*28*). Widespread fecal pollution of the surface waters was shown, yet the saltiness of the potable water supply after the disaster made much of the water unpalatable. Wells were vulnerable, perhaps to other etiologic agents of fecal origin including viruses and *Shigella* spp., with greater probability of infection than *Vibrio* spp., thus leading to the widespread diarrhea.

The most important lesson from the Asian tsunami is that disease epidemics can be prevented by public health intervention. Unfortunately, most flooding events, and other conditions that promote infectious disease epidemics, do not receive the same global media attention. A tsunami captures the imagination of the world in a way that weeks of rainfall in the Sudan or a rise in sea surface temperature cannot. However, if climatologic data can be used to predict future disease outbreaks, public health interventions can be mobilized in a more timely and proactive manner.

A continuing concern is the conditions that result in newly emergent virulent strains of pathogens. Faruque et al. have provided molecular evidence that *V. cholerae* O139 strains are derived from O1 strains through genetic modification (*29*). In addition, Chakraborty et al. in Kolkata have seen the presence and expression of virulence genes in several environmental strains of *V. cholerae* cul-

tured from surface waters (*30*). Recently, *E. coli* O157 has been isolated from the Ganges River in India for the first time (*31*). Indications are that it is metabolically different from *E. coli* O157 isolated from other parts of the world, but the conditions that have led to these differences are as yet unclear. From the above studies, risk for transmission of virulence genes is likely to be high, but studies of conditions promoting transmission and approaches to modeling resultant disease risks are in their infancy. New epidemic strains could potentially occur through mutation of existing epidemic strains or through gene transfer. Environmental stressors such as chemical contaminants are thought to accelerate both mutation rates and gene transfer (*32*). Thus, the degree of chemical pollution may need to be a component of disease models (in addition to other stressors).

The scientific community is a long way from incorporating environment-gene interactions into predictive models and clarifying the risks posed to human society from emerging diseases. However, investigation of these parts of the pathogen's ecology should remain on the national research agenda as we move forward with developing predictive models of disease outbreaks.

Current modeling of infectious diseases is by necessity retrospective. Environmental parameters measured by remote satellite imaging show the greatest promise for providing global coverage of changing environmental conditions. With current imaging technologies, we can measure sea surface temperature, sea surface height, chlorophyll A levels, and a variety of vegetation and soil indices, in addition to many other physical, biologic, and chemical parameters of the earth's surface and atmosphere. A variety of these parameters can be incorporated in complex mathematical models, together with biotic and ecologic variables of the pathogen and host life cycles, to correlate environment with outbreaks of disease (Figure 2). However, we are still far from being able to accurately predict future disease events on the basis of existing environmental conditions.

Successful predictive modeling of disease and the establishment of early warning systems have reached a critical junction in development. As we improve our understanding of the biology and ecology of the pathogen, vectors, and hosts, our ability to accurately link environmental variables, particularly those related to climate change, will improve. What has become clear over the past few years is that satellite imaging can play a critical role in disease prediction and, therefore, inform our response to future outbreaks.

We conclude that infectious disease events may be closely linked to environmental and global change. Satellite imaging may be critical for effective disease prediction and thus future mitigation of epidemic and pandemic diseases. We cannot stress too strongly our belief that a strong global satellite program is essential for future disease prediction.

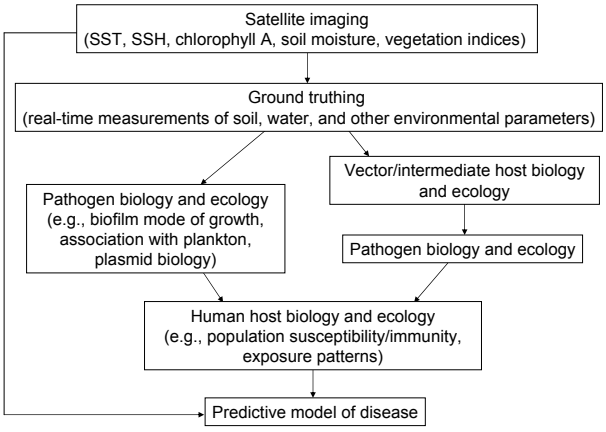

Figure 2. Components of a predictive model of infectious disease based on satellite imaging to assess environmental change. SST, sea surface temperature; SSH, sea surface height.

Acknowledgments

This article is dedicated to Terry L. Yates, an outstanding scientist and colleague whose substantial contributions to vector-borne and zoonotic disease research, in particular, his work on the ecology of hantavirus, will always be remembered.

T.E.F. was supported in part by grant no. P20 RR-16455-06 from the National Center for Research Resources, a component of the National Institutes of Health (NIH). S.M.M. was supported by Centers for Disease Control and Prevention cooperative agreements A1010-21/21, U90/CCU224241 (Centers for Public Health Preparedness) and U01/CI000442, the Arts and Letters Foundation, and NIH/National Institute for Allergy and Infectious Disease cooperative agreement 5U54AI057158 (Northeast Biodefense Center Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research). R.R.C. was supported in part by grant no. S0660009 from the National Oceanic and Atmospheric Administration

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References

1. Climate change 2007: synthesis report. Contribution of working groups I, II and III to the fourth assessment report of the Intergovernmental Panel on Climate Change. Geneva: Intergovernmental Panel on Climate Change; 2007 [cited 2008 Apr 19]. Available from http://www.ipcc.ch/pdf/assessment-report/ar4/syr/ar4_syr.pdf

2. Morens DM, Folkers GK, Fauci AS. The challenge of emerging and re-emerging infectious diseases. Nature. 2004;430:242–9. DOI: 10.1038/nature02759
3. Wilson ME, Levins R, Spielman A, editors. Disease in evolution: global changes and emergence of infectious diseases. New York: New York Academy of Sciences; 1994.
4. Curriero FC, Patz JA, Rose JB, Lele S. Analysis of the association between extreme precipitation and waterborne disease outbreaks in the United States, 1948–1994. Am J Public Health. 2001;91:1194–9. DOI: 10.2105/AJPH.91.8.1194
5. Hoyois P, Scheuren J-M, Below R, Guha-Sapin D. Annual disaster statistical review: numbers and trends, 2006. Brussels: Center for Research on the Epidemiology of Disasters, School of Public Health, Catholic University of Louvain; 2007 [cited 2008 Apr 19]. Available from http://www.emdat.be/Documents/Publications/Annual%20Disaster%20Statistical%20Review%202006.pdf
6. Sidley P. Floods in southern Africa result in cholera outbreak and displacement. BMJ. 2008;336:471. DOI: 10.1136/bmj.39503.700903.DB
7. Sur D, Dutta P, Nair GB, Bhattacharya SK. Severe cholera outbreak following floods in a northern district of West Bengal. Indian J Med Res. 2000;112:178–82.
8. Climate change 2001: synthesis report. A contribution of working groups I, II, and III to the third assessment report of the Intergovernmental Panel on Climate Change. Cambridge: Cambridge University Press; 2001 [cited 2008 Apr 19]. Available from http://www.ipcc.ch/ipccreports/tar/vol4/english/002.htm
9. Gil AI, Louis VR, Rivera ING, Lipp E, Huq A, Lanata CF. Occurrence and distribution of *Vibrio cholerae* in the coastal environment of Peru. Environ Microbiol. 2004;6:699–706. DOI: 10.1111/j.1462-2920.2004.00601.x
10. Colwell RR, Huq A, Islam MS, Aziz KMA, Yunus M, Khan NH, et al. Reduction of cholera in Bangladeshi villages by simple filtration. Proc Natl Acad Sci U S A. 2003;100:1051–5. DOI: 10.1073/pnas.0237386100
11. Šumilo D, Bormane A, Asokliene L, Lucenko I, Vasilenko V, Randolph S. Tick-borne encephalitis in the Baltic States: identifying risk factors in space and time. Int J Med Microbiol. 2006;296:76–9. DOI: 10.1016/j.ijmm.2005.12.006
12. Šumilo D, Asokliene L, Bormane A, Vasilenko V, Golovljova I, Randolph S. Climate change cannot explain the upsurge of tick-borne encephalitis in the Baltics. PLoS One. 2007;2:e500. DOI: 10.1371/journal.pone.0000500
13. Rogers DJ, Randolph SE, Snow RW, Hay SI. Satellite imagery in the study and forecast of malaria. Nature. 2002;415:710–5. DOI: 10.1038/415710a
14. Engelthaler DM, Mosley DG, Cheek JE, Levy CE, Komatsu KK, Ettestad P, et al. Climatic and environmental patterns associated with hantavirus pulmonary syndrome, Four Corners region, United States. Emerg Infect Dis. 1999;5:87–94.
15. Glass GE, Cheek JE, Patz JA, Shields TM, Doyle TJ, Thoroughman DA, et al. Using remotely sensed data to identify areas at risk for hantavirus pulmonary syndrome. Emerg Infect Dis. 2000;6:238–47.
16. Glass GE, Yates TL, Fine JB, Shields TM, Kendall JB, Hope AG, et al. Satellite imagery characterizes local animal reservoir populations of Sin Nombre virus in the southwestern United States. Proc Natl Acad Sci U S A. 2002;99:16817–22. DOI: 10.1073/pnas.252617999
17. Glass GE, Shields TM, Parmenter RR, Goade D, Mills JN, Cheek J, et al. Predicted hantavirus risk in 2006 for the southwestern U.S. Occasional Papers of the Museum of Texas Tech University. 2006;255:1–16.
18. First hantavirus case reported for 2006—Coloradans urged to take precautions. Boulder (CO): Colorado Department of Public Health and Environment; 2006 [cited 2008 Apr 19]. Available from http://www.bouldercounty.org/health/pr/2006/05112006hantaVirus.htm
19. Greenwood BM, Blakebrough IS, Bradley AK, Wali S, Whittle HC. Meningococcal disease and season in sub-Saharan Africa. Lancet. 1984;1:1339–42. DOI: 10.1016/S0140-6736(84)91830-0
20. Dowell SF, Ho MS. Seasonality of infectious diseases and severe acute respiratory syndrome—what we don't know can hurt us. Lancet Infect Dis. 2004;4:704–8. DOI: 10.1016/S1473-3099(04)01177-6
21. Chew FT, Doraisingham S, Ling AE, Kumarasinghe G, Lee BW. Seasonal trends of viral respiratory tract infections in the tropics. Epidemiol Infect. 1998;121:121–8. DOI: 10.1017/S0950268898008905
22. Broutin H, Philippon S, Constantin de Magny G, Courel M-F, Sultan B, Guégan JF. Comparative study of meningitis dynamics across nine African countries: a global perspective. Int J Health Geographics. 2007;6:29 [cited 2009 Mar 20]. Available from http://www.ij-healthgeographics.com/content/6/1/29
23. Xiao X, Boles S, Frolking SE, Li C, Babu JY, Salas W, et al. Mapping paddy rice agriculture in south and Southeast Asia using multi-temporal MODros Inf Serv. images. Remote Sens Environ. 2006;100:95–113. DOI: 10.1016/j.rse.2005.10.004
24. Kilpatrick AM. Chmura AA, Gibbons DW, Fleischer RC, Marra PP, Daszak P. Predicting the global spread of H5N1 avian influenza. Proc Natl Assoc Sci. 2006;103:19368–73. DOI: 10.1073/pnas.0609227103
25. Gilbert M, Xiao X, Domenech J, Lubroth J, Martin V, Slingenberg J. Anatidae migration in the western Palearctic and spread of highly pathogenic avian influenza H5N1 virus. Emerg Infect Dis. 2006;12:1650–6.
26. Sims D. UNH Research uses satellite observation to track avian flu. Durham (NH): University of New Hampshire Media Relations; November 20, 2006 [cited 2008 Apr 19]. Available from http://www.unh.edu/news/cj_nr/2006/nov/ds20flu.cfm
27. Brennan RJ, Rimba K. Rapid health assessment in Aceh Jaya District, Indonesia, following the December 26 tsunami. Emerg Med Australas. 2005;17:341–50. DOI: 10.1111/j.1742-6723.2005.00755.x
28. Jamieson C. Preventing the second wave. Operation Sumatra Assist, Australian Government, Department of Defense; 2005 [cited 2008 Apr 18]. Available from http://defence.gov.au/optsunamiassist/news/article045/index.htm
29. Faruque SM, Albert MJ, Mekalanos JJ. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol Mol Biol Rev. 1998;62:1301–14.
30. Chakraborty S, Mukhopadhyay AK, Bhadra RK, Ghosh AN, Mitra R, Shimada T, et al. Virulence genes in environmental strains of *Vibrio cholerae*. Appl Environ Microbiol. 2000;66:4022–8. DOI: 10.1128/AEM.66.9.4022-4028.2000
31. Hamner S, Broadaway SC, Mishra VB, Tripathi A, Mishra RK, Pulcini E, et al. Isolation of potentially pathogenic *Escherichia coli* O157:H7 from the Ganges River. Appl Environ Microbiol. 2007;73:2369–72. DOI: 10.1128/AEM.00141-07
32. Martinez RJ, Wang Y, Raimondo MA, Coombs JM, Barkay T, Sobecky PA. Horizontal gene transfer of PIB-type ATPases among bacteria isolated from radionuclide- and metal-contaminated subsurface soils. Appl Environ Microbiol. 2006;72:3111–8. DOI: 10.1128/AEM.72.5.3111-3118.2006

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Zika Virus Outside Africa

Edward B. Hayes

Zika virus (ZIKV) is a flavivirus related to yellow fever, dengue, West Nile, and Japanese encephalitis viruses. In 2007 ZIKV caused an outbreak of relatively mild disease characterized by rash, arthralgia, and conjunctivitis on Yap Island in the southwestern Pacific Ocean. This was the first time that ZIKV was detected outside of Africa and Asia. The history, transmission dynamics, virology, and clinical manifestations of ZIKV disease are discussed, along with the possibility for diagnostic confusion between ZIKV illness and dengue. The emergence of ZIKV outside of its previously known geographic range should prompt awareness of the potential for ZIKV to spread to other Pacific islands and the Americas.

In April 2007, an outbreak of illness characterized by rash, arthralgia, and conjunctivitis was reported on Yap Island in the Federated States of Micronesia. Serum samples from patients in the acute phase of illness contained RNA of Zika virus (ZIKV), a flavivirus in the same family as yellow fever, dengue, West Nile, and Japanese encephalitis viruses. These findings show that ZIKV has spread outside its usual geographic range (*1,2*).

Sixty years earlier, on April 18, 1947, fever developed in a rhesus monkey that had been placed in a cage on a tree platform in the Zika Forest of Uganda (*3*). The monkey, Rhesus 766, was a sentinel animal in the Rockefeller Foundation's program for research on jungle yellow fever. Two days later, Rhesus 766, still febrile, was brought to the Foundation's laboratory at Entebbe and its serum was inoculated into mice. After 10 days all mice that were inoculated intracerebrally were sick, and a filterable transmissible agent, later named Zika virus, was isolated from the mouse brains. In early 1948, ZIKV was also isolated from *Aedes africanus* mosquitoes trapped in the same forest (*4*). Serologic studies indicated that humans could also be infected (*5*). Transmission of ZIKV by artificially fed *Ae. aegypti* mosquitoes to mice and a monkey in a laboratory was reported in 1956 (*6*).

ZIKV was isolated from humans in Nigeria during studies conducted in 1968 and during 1971–1975; in 1 study, 40% of the persons tested had neutralizing antibody to ZIKV (*7–9*). Human isolates were obtained from febrile children 10 months, 2 years (2 cases), and 3 years of age, all without other clinical details described, and from a 10 year-old boy with fever, headache, and body pains (*7,8*). From 1951 through 1981, serologic evidence of human ZIKV infection was reported from other African countries such as Uganda, Tanzania, Egypt, Central African Republic, Sierra Leone (*10*), and Gabon, and in parts of Asia including India, Malaysia, the Philippines, Thailand, Vietnam, and Indonesia (*10–14*). In additional investigations, the virus was isolated from *Ae. aegypti* mosquitoes in Malaysia, a human in Senegal, and mosquitoes in Côte d'Ivoire (*15–17*). In 1981 Olson et al. reported 7 people with serologic evidence of ZIKV illness in Indonesia (*11*). A subsequent serologic study indicated that 9/71 (13%) human volunteers in Lombok, Indonesia, had neutralizing antibody to ZIKV (*18*). The outbreak on Yap Island in 2007 shows that ZIKV illness has been detected outside of Africa and Asia (Figure 1).

Dynamics of Transmission

ZIKV has been isolated from *Ae. africanus*, *Ae. apicoargenteus*, *Ae. luteocephalus*, *Ae. aegypti*, *Ae vitattus*, and *Ae. furcifer* mosquitoes (*9,15,17,19*). *Ae. hensilii* was the predominant mosquito species present on Yap during the ZIKV disease outbreak in 2007, but investigators were unable to detect ZIKV in any mosquitoes on the island during the outbreak (*2*). Dick noted that *Ae. africanus* mosquitoes, which were abundant and infected with ZIKV in the Zika Forest, were not likely to enter monkey cages such as

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DOI: 10.3201/eid1509.090442

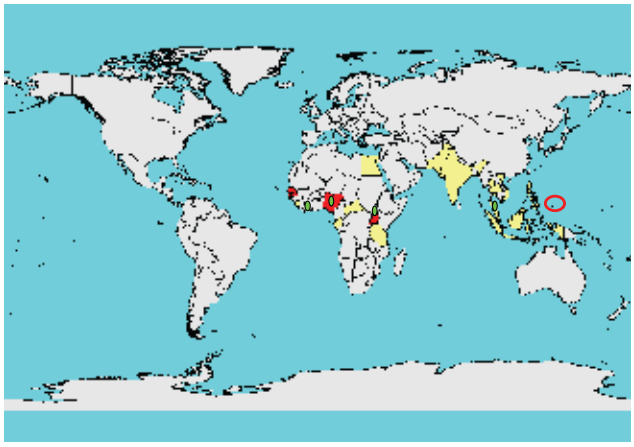

Figure 1. Approximate known distribution of Zika virus, 1947–2007. Red circle represents Yap Island. Yellow indicates human serologic evidence; red indicates virus isolated from humans; green represents mosquito isolates.

the one containing Rhesus 766 (*5*) raising the doubt that the monkey might have acquired ZIKV from some other mosquito species or through some other mechanism. During the studies of yellow fever in the Zika Forest, investigators had to begin tethering monkeys in trees because caged monkeys did not acquire yellow fever virus when the virus was present in mosquitoes (*5*). Thus, despite finding ZIKV in *Ae. Africanus* mosquitoes, Dick was not sure whether or not these mosquitoes were actually the vector for enzootic ZIKV transmission to monkeys.

Boorman and Porterfield subsequently demonstrated transmission of ZIKV to mice and monkeys by *Ae. aegypti* in a laboratory (*6*). Virus content in the mosquitoes was high on the day of artificial feeding, dropped to undetectable levels through day 10 after feeding, had increased by day 15, and remained high from days 20 through 60 (*6*). Their study suggests that the extrinsic incubation period for ZIKV in mosquitoes is ≈10 days. The authors cautioned that their results did not conclusively demonstrate that *Ae. aegypti* mosquitoes could transmit ZIKV at lower levels of viremia than what might occur among host animals in natural settings. Nevertheless, their results, along with the viral isolations from wild mosquitoes and monkeys and the phylogenetic proximity of ZIKV to other mosquito-borne flaviviruses, make it reasonable to conclude that ZIKV is transmitted through mosquito bites.

There is to date no solid evidence of nonprimate reservoirs of ZIKV, but 1 study did find antibody to ZIKV in rodents (*20*). Further laboratory, field, and epidemiologic studies would be useful to better define vector competence for ZIKV, to determine if there are any other arthropod vectors or reservoir hosts, and to evaluate the possibility of congenital infection or transmission through blood transfusion.

Virology and Pathogenesis

ZIKV is an RNA virus containing 10,794 nucleotides encoding 3,419 amino acids. It is closely related to Spondweni virus; the 2 viruses are the only members of their clade within the mosquito-borne cluster of flaviviruses (Figure 2) (*1,21,22*). The next nearest relatives include Ilheus, Rocio, and St. Louis encephalitis viruses; yellow fever virus is the prototype of the family, which also includes dengue, Japanese encephalitis, and West Nile viruses (*1,21*). Studies in the Zika Forest suggested that ZIKV infection blunted the viremia caused by yellow fever virus in monkeys but did not block transmission of yellow fever virus (*19,23*).

Information regarding pathogenesis of ZIKV is scarce but mosquito-borne flaviviruses are thought to replicate initially in dendritic cells near the site of inoculation then spread to lymph nodes and the bloodstream (*24*). Although flaviviral replication is thought to occur in cellular cytoplasm, 1 study suggested that ZIKV antigens could be found in infected cell nuclei (*25*). To date, infectious ZIKV has been detected in human blood as early as the day of illness onset; viral nucleic acid has been detected as late as 11 days after onset (*1,26*). The virus was isolated from the serum of a monkey 9 days after experimental inoculation (*5*). ZIKV is killed by potassium permanganate, ether, and temperatures >60°C, but it is not effectively neutralized with 10% ethanol (*5*).

Clinical Manifestations

The first well-documented report of human ZIKV disease was in 1964 when Simpson described his own occupationally acquired ZIKV illness at age 28 (*27*). It began with mild headache. The next day, a maculopapular rash covered his face, neck, trunk, and upper arms, and spread to his palms and soles. Transient fever, malaise, and back pain developed. By the evening of the second day of illness he was afebrile, the rash was fading, and he felt better. By day three, he felt well and had only the rash, which disappeared over the next 2 days. ZIKV was isolated from serum collected while he was febrile.

In 1973, Filipe et al. reported laboratory-acquired ZIKV illness in a man with acute onset of fever, headache, and joint pain but no rash (*26*). ZIKV was isolated from serum collected on the first day of symptoms; the man's illness resolved in ≈1 week.

Of the 7 ZIKV case-patients in Indonesia described by Olson et al. all had fever, but they were detected by hospital-based surveillance for febrile illness (*11*). Other manifestations included anorexia, diarrhea, constipation, abdominal pain, and dizziness. One patient had conjunctivitis but none had rash. The outbreak on Yap Island was characterized by rash, conjunctivitis, and arthralgia (*1,2*). Other less frequent manifestations included myalgia, headache, retroorbital pain, edema, and vomiting (*2*).

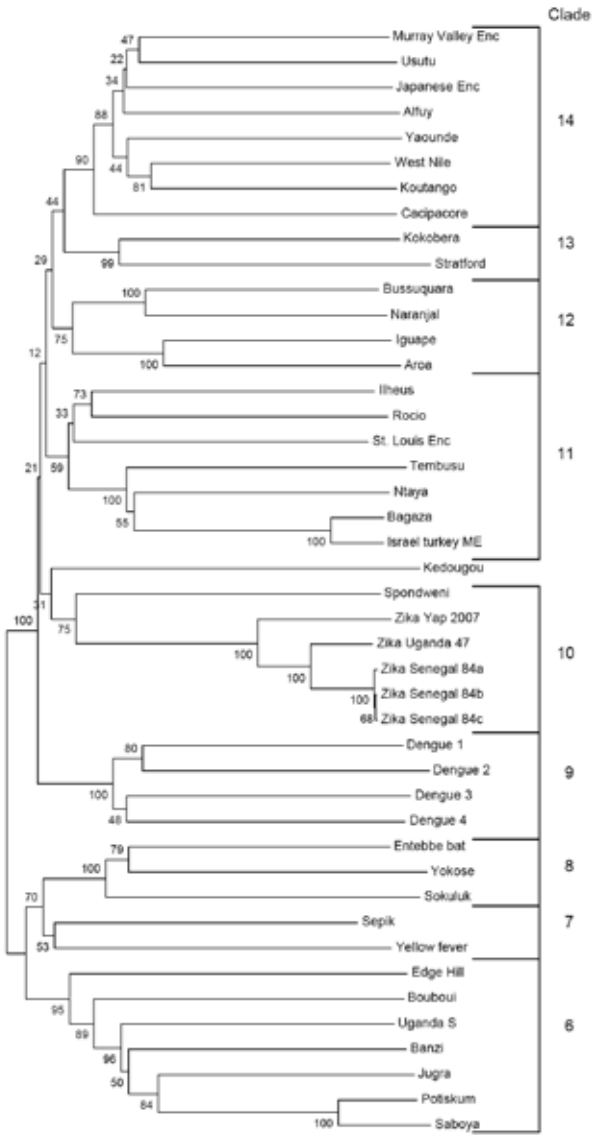

Figure 2. Phylogenetic relationship of Zika virus to other flaviviruses based on nucleic acid sequence of nonstructural viral protein 5, with permission from Dr Robert Lanciotti (*1*). Enc, encephalitis; ME, meningoencephalitis.

Diagnosis

Diagnostic tests for ZIKV infection include PCR tests on acute-phase serum samples, which detect viral RNA, and other tests to detect specific antibody against ZIKV in serum. An ELISA has been developed at the Arboviral Diagnostic and Reference Laboratory of the Centers for Disease Control and Prevention (Atlanta, GA, USA) to detect immunoglobulin (Ig) M to ZIKV (*1*). In the samples from Yap Island, cross-reactive results in sera from convalescent-phase patients occurred more frequently among patients with evidence of previous flavivirus infections than among those with apparent primary ZIKV infections (*1,2*). Cross-reactivity was more frequently noted with dengue virus than with yellow fever, Japanese encephalitis, Murray Valley encephalitis, or West Nile viruses, but there were too few samples tested to derive robust estimates of the sensitivity and specificity of the ELISA. IgM was detectable as early as 3 days after onset of illness in some persons; 1 person with evidence of previous flavivirus infection had not developed IgM at day 5 but did have it by day 8 (*1*). Neutralizing antibody developed as early as 5 days after illness onset. The plaque reduction neutralization assay generally has improved specificity over immunoassays, but may still yield cross-reactive results in secondary flavivirus infections. PCR tests can be conducted on samples obtained less than 10 days after illness onset; 1 patient from Yap Island still had detectable viral RNA on day 11 (*1*). In general, diagnostic testing for flavivirus infections should include an acute-phase serum sample collected as early as possible after onset of illness and a second sample collected 2 to 3 weeks after the first.

Public Health Implications

Because the virus has spread outside Africa and Asia, ZIKV should be considered an emerging pathogen. Fortunately, ZIKV illness to date has been mild and self-limited, but before West Nile virus caused large outbreaks of neuroinvasive disease in Romania and in North America, it was also considered to be a relatively innocuous pathogen (*28*). The discovery of ZIKV on the physically isolated community of Yap Island is testimony to the potential for travel or commerce to spread the virus across large distances. A medical volunteer who was on Yap Island during the ZIKV disease outbreak became ill and was likely viremic with ZIKV after her return to the United States (*2*). The competence of mosquitoes in the Americas for ZIKV is not known and this question should be addressed. Spread of ZIKV across the Pacific could be difficult to detect because of the cross-reactivity of diagnostic flavivirus antibody assays. ZIKV disease could easily be confused with dengue and might contribute to illness during dengue outbreaks. Recognition of the spread of ZIKV and of the impact of ZIKV on human health will require collaboration between clinicians, public health officials, and high-quality reference laboratories.

Given that the epidemiology of ZIKV transmission on Yap Island appeared to be similar to that of dengue, strategies for prevention and control of ZIKV disease should include promoting the use of insect repellent and interventions to reduce the abundance of potential mosquito vectors. Officials responsible for public health surveillance in the Pacific region and the United States should be alert to the potential spread of ZIKV and keep in mind the possible diagnostic confusion between ZIKV illness and dengue.

Acknowledgment

The author thanks Marc Fischer for helpful comments on the manuscript.

Dr Hayes is currently a research professor at the Barcelona Centre for International Health Research. His research interests include epidemiology and prevention of vector-borne infectious diseases, and evaluation of safety and effectiveness of preventive interventions.

References

1. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14:1232–9. DOI: 10.3201/eid1408.080287
2. Duffy MR, Chen T, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360:2536–43. DOI: 10.1056/NEJMoa0805715
3. Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952;46:509–20. DOI: 10.1016/0035-9203(52)90042-4
4. Macnamara FN. Zika virus: a report on three cases of human infection during an epidemic of jaundice in Nigeria. Trans R Soc Trop Med Hyg. 1954;48:139–45. DOI: 10.1016/0035-9203(54)90006-1
5. Dick GW. Zika virus. II. Pathogenicity and physical properties. Trans R Soc Trop Med Hyg. 1952;46:521–34. DOI: 10.1016/0035-9203(52)90043-6
6. Boorman JP, Porterfield JS. A simple technique for infection of mosquitoes with viruses; transmission of Zika virus. Trans R Soc Trop Med Hyg. 1956;50:238–42. DOI: 10.1016/0035-9203(56)90029-3
7. Moore DL, Causey OR, Carey DE, Reddy S, Cooke AR, Akinkugbe FM, et al. Arthropod-borne viral infections of man in Nigeria, 1964–1970. Ann Trop Med Parasitol. 1975;69:49–64.
8. Fagbami A. Epidemiological investigations on arbovirus infections at Igbo-Ora, Nigeria. Trop Geogr Med. 1977;29:187–91.
9. Fagbami AH. Zika virus infections in Nigeria: virological and seroepidemiological investigations in Oyo State. J Hyg (Lond). 1979;83:213–9. DOI: 10.1017/S0022172400025997
10. Robin Y, Mouchet J. Serological and entomological study on yellow fever in Sierra Leone. Bull Soc Pathol Exot Filiales. 1975;68:249–58.
11. Olson JG, Ksiazek TG. Suhandiman, Triwibowo. Zika virus, a cause of fever in Central Java, Indonesia. Trans R Soc Trop Med Hyg. 1981;75:389–93. DOI: 10.1016/0035-9203(81)90100-0
12. Jan C, Languillat G, Renaudet J, Robin Y. A serological survey of arboviruses in Gabon [in French]. Bull Soc Pathol Exot Filiales. 1978;71:140–6.
13. Saluzzo JF, Gonzalez JP, Hervé JP, Georges AJ. Serological survey for the prevalence of certain arboviruses in the human population of the south-east area of Central African Republic [in French]. Bull Soc Pathol Exot Filiales. 1981;74:490–9.
14. Saluzzo JF, Ivanoff B, Languillat G, Georges AJ. Serological survey for arbovirus antibodies in the human and simian populations of the South-East of Gabon [in French]. Bull Soc Pathol Exot Filiales. 1982;75:262–6.
15. Marchette NJ, Garcia R, Rudnick A. Isolation of Zika virus from Aedes aegypti mosquitoes in Malaysia. Am J Trop Med Hyg. 1969;18:411–5.
16. Monlun E, Zeller H, Le Guenno B, Traoré-Lamizana M, Hervy JP, Adam F, et al. Surveillance of the circulation of arbovirus of medical interest in the region of eastern Senegal [in French]. Bull Soc Pathol Exot. 1993;86:21–8.
17. Akoua-Koffi C, Diarrassouba S, Bénié VB, Ngbichi JM, Bozoua T, Bosson A, et al. Investigation surrounding a fatal case of yellow fever in Côte d'Ivoire in 1999 [in French]. Bull Soc Pathol Exot. 2001;94:227–30.
18. Olson JG, Ksiazek TG, Gubler DJ, Lubis SI, Simanjuntak G, Lee VH, et al. A survey for arboviral antibodies in sera of humans and animals in Lombok, Republic of Indonesia. Ann Trop Med Parasitol. 1983;77:131–7.
19. McCrae AW, Kirya BG. Yellow fever and Zika virus epizootics and enzootics in Uganda. Trans R Soc Trop Med Hyg. 1982;76:552–62. DOI: 10.1016/0035-9203(82)90161-4
20. Darwish MA, Hoogstraal H, Roberts TJ, Ahmed IP, Omar F. A sero-epidemiological survey for certain arboviruses (Togaviridae) in Pakistan. Trans R Soc Trop Med Hyg. 1983;77:442–5. DOI: 10.1016/0035-9203(83)90106-2
21. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB. Phylogeny of the genus *Flavivirus*. J Virol. 1998;72:73–83.
22. Cook S, Holmes EC. A multigene analysis of the phylogenetic relationships among the flaviviruses (family: Flaviviridae) and the evolution of vector transmission. Arch Virol. 2006;151:309–25. DOI: 10.1007/s00705-005-0626-6
23. Haddow AJ, Williams MC, Woodall JP, Simpson DI, Goma LK. Twelve isolations of Zika virus from *Aedes (Stegomyia) africanus* (Theobald) taken in and above a Uganda forest. Bull World Health Organ. 1964;31:57–69.
24. Diamond MS, Shrestha B, Mehlhop E, Sitati E, Engle M. Innate and adaptive immune responses determine protection against disseminated infection by West Nile encephalitis virus. Viral Immunol. 2003;16:259–78. DOI: 10.1089/088282403322396082
25. Buckley A, Gould EA. Detection of virus-specific antigen in the nuclei or nucleoli of cells infected with Zika or Langat virus. J Gen Virol. 1988;69:1913–20. DOI: 10.1099/0022-1317-69-8-1913
26. Filipe AR, Martins CM, Rocha H. Laboratory infection with Zika virus after vaccination against yellow fever. Arch Gesamte Virusforsch. 1973;43:315–9. DOI: 10.1007/BF01556147
27. Simpson DI. Zika virus infection in man. Trans R Soc Trop Med Hyg. 1964;58:335–8. DOI: 10.1016/0035-9203(64)90201-9
28. Petersen LR, Hayes EB. Westward ho?—the spread of West Nile virus. N Engl J Med. 2004;351:2257–9. DOI: 10.1056/NEJMp048261

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Extrapulmonary Infections Associated with Nontuberculous Mycobacteria in Immunocompetent Persons

Claudio Piersimoni and Claudio Scarparo

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Learning Objectives

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

- Diagnose and treat nontuberculous mycobacterial (NTM) lymphadenitis effectively
- Identify elements of NTM osteoarticular infections
- Treat NTM skin infections according to standards of care
- Describe infections with rapidly growing mycobacteria

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Disclosures: ***Claudio Piersimoni, MD*****;** *and* ***Claudio Scarparo, MD*****,** *have disclosed no relevant financial relationships.*

Over the past several years, the prevalence of human disease caused by nontuberculous mycobacteria (NTM) has increased. Whether the increase in cases is real or whether more cases are being recognized remains unclear. Despite a considerable increase in knowledge about NTM infections, they still represent a diagnostic and therapeutic challenge for several reasons: 1) pathogenic isolates may be indistinguishable from contaminant or saprophytic isolates; 2) timely and reliable identification of isolates may depend on proper communication between clinicians and laboratory staff; 3) lack of standardized susceptibility testing makes adoption of tailored therapies unrealistic; and 4) lack of treatment guidelines exposes patients to toxic drugs and disappointing outcomes. Laboratory research and multicenter controlled trials are needed to improve diagnosis and treatment of these infections.

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DOI: 10.3201/eid1509.081259

The >120 recognized species of nontuberculous mycobacteria (NTM) share common features: 1) they are facultative pathogens; 2) evidence of human-to-human transmission is lacking; 3) some NTM species are ubiquitous and others have more restricted distribution; 4) treatment may be difficult and vary according to the involved organism and disease site; and 5) pathogenesis is still undefined, depending on the interaction between the microorganism and the host's immune system (*1*). About 90% of cases involve the pulmonary system; the rest involve lymph nodes, skin, soft tissues, and bones. Less frequently

reported are central nervous system disease, keratitis, and otitis media (*1*,*2*). We reviewed the epidemiology, clinical features, diagnosis, and treatment of the most common extrapulmonary diseases associated with NTM in immunocompetent persons (*2*–*5*).

Lymphadenitis

Localized lymphadenitis most commonly affects children; peak incidence occurs at 1–5 years of age (*6*). The route of infection is hypothesized to be by way of the lymphatic vessels that drain the mouth and pharynx. The most frequently isolated species is *Mycobacterium avium* complex (MAC), followed by *M. scrofulaceum*, *M. malmoense,* and *M. hemophilum* (*7*). However, a growing number of previously unrecognized slow-growing mycobacteria have been implicated with increasing frequency in reports of isolated or microclustered cases (Table 1) (*8*).

Generally, NTM adenitis is an indolent disease; most patients are otherwise healthy and have as their sole clinical sign a chronic neck mass that does not respond to antimicrobial drug therapy. The disease is usually unilateral and occurs in the cervical, submandibular, or preauricular lymph nodes, although parotid and postauricular node involvement has been reported. The nodes enlarge and may rapidly soften and rupture, forming a draining sinus. Although spontaneous regression has occasionally been described, healing usually occurs by fibrosis and calcification. Pyogenic and tuberculous adenitis are the most important differential diagnoses.

Although a presumptive diagnosis of nontuberculous mycobacterial adenitis can be made on the basis of clinical history and physical examination, definitive diagnosis depends upon the recovery of mycobacteria. Every effort should be made to obtain material for culture and further identification. Cultures of draining and ulcerated lesions, especially when swabs are used for specimen sampling, have been shown to give a lower diagnostic yield than do needle aspirates (*9*) or tissue biopsy samples. For recovery of NTM, use of a liquid medium or radiometric growth detection are regarded as the standard. Moreover, in children who have not been vaccinated with *M. bovis* BCG, purified protein derivative skin testing may be used as a surrogate test to diagnose chronic cervicofacial lymphadenitis (*10*). Histologic appearance of necrotizing granulomatous inflammation with various degrees of caseation is also diagnostic.

Table 1. Less frequently encountered mycobacterial species recovered from immunocompetent persons with lymphadenitis*

Mycobacterium sp.	No. cases reported	Identification method
M. bohemicum	3	GS
M. celatum	1	RH, HPLC, GS
M. genavense	1	RH, GS
M. heckeshornense	1	GS
M. heidelbergense	1	GS
M. interjectum	4	GS
M. lentiflavum	5	HPLC, GS
M. palustre	1	GS
M. parmense	1	HPLC, GS
M. simiae	4	RH, HPLC, GS
M. triplex	4	GS
M. tusciae	1	HPLC, GS

*Most persons were children. GS, gene sequencing; RH, reverse hybridization; HPLC, high-performance liquid chromatography.

Treatment of uncomplicated NTM lymphadenitis is complete surgical excision. Incision and drainage are discouraged because they usually lead to sinus tract formation with chronic discharge. In a recent well-designed trial including 100 children with culture- or PCR-confirmed diagnoses, surgery was more effective than chemotherapy; cure rates were 96% and 66%, respectively (*11*). Total excision should be performed as early as possible to maximize recovery of the causative agent, to prevent further cosmetic damage, to prevent extensive spread and subsequently more difficult excision, and to cure disease. For some patients, however, surgery is associated with substantial risk either because of a discharging sinus or proximity of facial nerve branches. For these patients, chemotherapy preceded by a diagnostic biopsy sample is recommended. Chemotherapy should also be considered for patients in whom lymphadenitis recurs after surgery or for whom all abnormal tissue could not be excised. Recent data indicate fine-needle aspiration as the preferred diagnostic technique for patients with nontuberculous mycobacterial adenitis who do not undergo surgical excision. The optimal chemotherapeutic regimen and its duration are still undetermined, but combination therapy including clarithromycin and a rifamycin, either rifampin or rifabutin and/or ethambutol, may be beneficial.

Osteoarticular Infections

NTM infections involving the musculoskeletal system are uncommon. However, when they do occur, both rapid- and slow-growing species have been implicated in chronic granulomatous infections involving tendon sheaths, bursae, bones, and joints. These are usually acquired by direct inoculation of the pathogen from an environmental source or a contiguous infection focus as a consequence of surgical procedures, penetrating trauma, injuries, or needle injections. Most affected patients are immunocompetent, but some mycobacterial species, such as *M. chelonae* and *M. hemophilum,* are almost entirely recovered from patients with serious underlying diseases (HIV infection, immunosuppressive therapy, or blood disorders) (*4*). In a recent study of vertebral osteomyelitis caused by NTM (*12*), various degrees of immunosuppression were found in 17 (51.5%) of 33 patients. From the 31 patients with spinal infection studied, the following NTM species were recovered: MAC (n = 13), *M. xenopi* (n = 7), *M. fortuitum* (n =

5), *M. abscessus* (n = 3), *M. kansasii* (n = 1), *M. simiae* (n = 1), and unidentified NTM species (n = 1). *M. hemophilum* also causes NTM osteomyelitis infections in bone marrow and solid organ transplants (*13*). The hand and wrist are the most frequently reported sites of NTM tenosynovitis because of their abundance of synovial fluid and tissue combined with a higher probability of penetrating injury. Most frequently, *M. marinum* and *M. kansasii* are involved; less frequently, *M. avium* complex, *M. szulgai*, *M. terrae*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. malmoense,* and *M. xenopi* are found (*14*).

Clinically, osteoarticular infections caused by NTM are indistinguishable from tuberculosis-associated infections. Signs and symptoms such as localized pain (with or without neurologic impairment), joint stiffness and swelling, low-grade fever, sweating, chills, anorexia, malaise, and weight loss have been reported (Table 2). On rare occasions, suppuration followed by extensive necrosis of the synovial tissue may occur (*3*), although in more severe cases infection may extend to the periosteum and lead to osteomyelitis (*3*). The clinical course of the disease is typically protracted; average time from onset of symptoms to diagnosis may be as long as 10 months. To prevent severe tissue destruction and neurologic disorders, prompt and accurate diagnosis is essential. Diagnosis relies on clinical suspicion and must be considered for patients with increasing musculoskeletal system signs, those with inflammation after penetrating or blunt trauma, and those with underlying risk factors who undergo a medical procedure. Culture of synovial fluid and tissue biopsy are mandatory for definitive diagnosis and identification of the causative agent. Computed tomography and sonography may help guide percutaneous tissue biopsy sampling of infected areas or diagnostic aspiration of intraarticular fluid (*15*). Histopathologic examination has shown a spectrum of inflammatory changes, including granulomatous lesions with or without caseation (*3*).

Lack of the following have greatly limited development of consensus guidelines for the treatment of musculoskeletal infections caused by NTM: correlation between in vitro susceptibility testing and clinical outcome, standardized antimicrobial-drug susceptibility testing for most NTM species, and clinical trials comparing different therapeutic regimens among an adequate number of patients. Prolonged chemotherapy with an isolate-tailored

Table 2. Clinical signs associated with osteoarticular infections caused by nontuberculous mycobacteria

Disease	Affected site	*Mycobacterium* spp.	Underlying diseases and risk factors (no. cases reported)
Arthritis, osteomyelitis	Thumb (interphalangeal joint)	*M. malmoense*	Rheumatoid arthritis (1)
Arthritis	Knee, ankle	*M. xenopi*	None* (6), invasive medical procedure (1)
Arthritis	Multiple joints: wrist, knee, finger, ankle, elbow, vertebrae, shoulder	*M. kansasii*	AIDS (13), rheumatoid arthritis (3), systemic lupus erythematous (2), renal transplant (2), polymyositis (1), progressive systemic sclerosis (1), myelodysplasia (1), none (26), localized trauma (10), steroid therapy (10)
Arthritis	Knee	*M. kansasii*	Prosthetic joint (1)
Synovitis (carpal tunnel syndrome)	Wrist	*M. szulgai*	No underlying disease, fish-tank cleaning (2)
Tenosynovitis	Hand	*M. intracellulare*	None* (1)
Tenosynovitis	Hand	*M. chelonae*	Penetrating injury (2), fracture (1), Immunosuppression (3)
Tenosynovitis	Hand, wrist	*M. avium* complex	Steroid injection (1), trauma (8), surgery (4)
Osteomyelitis	Sternum, foot, elbow	*M. wolinsky*	Cardiac surgery (1), stepped on nail (1), open fracture (1)
Osteomyelitis	Femur, tibia, calcaneus, toe, elbow, sternum	*M. goodii*	Open fracture (4), stepped on nail (1), surgery (2), penetrating trauma (2), puncture wound (1)
Osteomyelitis	Wrist	*M. scrofulaceum*	Diabetes (1)
Osteomyelitis	Hand, ankle, wrist	*M. marinum*	Fisherman exposed to aquarium (25), trauma (1), local or systemic steroids (20)
Osteomyelitis		*M. ulcerans*	Traumatic injury (213)
Osteomyelitis	Vertebrae	*M. avium complex, M. xenopi, M. fortuitum, M. abscessus, M. kansasii, M. simiae*	Systemic lupus erythematous and treatment with steroids (7), AIDS (4), interferon receptor defect (3), carcinoma (1), renal failure (1), chronic granulomatous disease (1), none* (16)
Osteomyelitis	Vertebrae	*M. xenopi*	Vertebral disk surgery (58)
Osteomyelitis	Tibia	*M. conceptionense*	Fracture (1)
Osteomyelitis	Vertebrae	*M. abscessus*	Trauma to the back (1)
Osteomyelitis	Vertebrae	*M. avium complex*	Trauma to the back (1)
Osteomyelitis	Lower extremity, upper extremity, vertebrae, disseminated disease	*M. hemophilum*	AIDS (21), bone marrow or solid organ transplant (7); AIDS plus solid organ transplant (1), lymphoma (2), polycythemia vera (1)

*Otherwise healthy with no underlying disease or risk factor.

drug combination associated with surgical debridement is currently recommended for all musculoskeletal infections, especially in patients with abscesses (*12*).

Skin and Soft Tissue Infections

Skin and soft tissue infections usually occur after traumatic injury, surgery, or cosmetic procedures, which may expose a wound to soil, water, or medical devices occasionally contaminated with environmental mycobacteria. Although the epidemiology and clinical presentations of NTM responsible for skin and soft tissue infections differ, some species (MAC, *M. kansasii*, *M. xenopi*, and *M. marinum*) have been reported worldwide, whereas others (*M. ulcerans*) have limited geographic distribution.

M. marinum causes diseases in many fish species and is distributed worldwide. It is an opportunistic pathogen of humans, in whom infection is infrequent and occurs by direct injury from fish fins or bites or after cutaneous trauma and subsequent exposure to contaminated water or other sources of infection (shrimp, shellfish, frogs, turtles, dolphin, eels, and oysters). An increasing number of cases have been reported from most countries with temperate climates (*1*). Predisposing occupations and activities include fishery worker, seafood handler, fish-tank owner, fisherman, pet shop worker, and water-related recreational exposure (*4*). Consistent with the organism's growth at low temperatures, *M. marinum* infections are usually limited to the skin and confined, with few exceptions, to 1 extremity. For fish-tank owners, disease is often located in the hand or fingers; for those who swim in pools, the elbow is affected for 85%, followed by knee and foot (*16*). The incubation period is usually <4 weeks but can be as long as 9 months. Signs and symptoms of early infection are nonspecific, e.g., swelling and pain followed by ≥1 skin lesions (*17*). At the inoculation site, an erythematous or bluish papulonodular lesion (≈0.5–3 cm) develops and slowly enlarges, becoming more tender until it suppurates (*18*) (Figure). In ≈33% of patients, *M. marinum* infection may spread to deeper structures (soft tissues, tendons, and bone) (*19*), leading to extensive scarring and varying degrees of functional impairment. Less frequently, the disease extends from the inoculation site to regional lymph nodes along the lymphatic vessels, mimicking the clinical appearance of cutaneous sporotrichosis (*20*). Key elements for diagnosis of *M. marinum* infection are a history of exposure to potential sources of infection; a histopathologic appearance of granulomatous inflammation but no caseation; and culture growth of *M. marinum*, which strongly depends on incubation temperature. For localized skin lesions without a history of exposure to fish tanks, swimming pools, or tropical fish, other NTM must be considered (*21*).

Although presumptive identification can rely on a few biochemical and phenotypic tests such as production of photochromogenic pigment, negative nitrate reduction, and positive tests for urease and Tween 80 hydrolysis, definitive identification involves reverse hybridization techniques, or, alternatively, high-performance liquid chromatography analysis of mycolic acids and DNA sequencing assays (*22,23*). In vitro susceptibility test results of *M. marinum* clinical isolates have been reported extensively in the literature. Only clarithromycin, minocycline, and amikacin provide complete coverage (100% susceptibility); doxycycline, rifampin, and trimethoprim/sulfamethoxazole encounter different degrees of resistance (*24*). Drug-susceptibility testing of *M. marinum* isolates is recommended only for patients who remain culture positive after several months of therapy (*25*). Although a standardized regimen for *M. marinum* disease is still undefined, monotherapy with doxycycline, minocycline, trimethoprim/sulfamethoxazole, or clarithromycin should be limited to patients with mild disease only. Clarithromycin combined with ethambutol or rifampin is likely the best combination therapy. Treatment with 2 agents should be continued for at least 1–2 months after resolution of skin lesions. In addition, surgical treatment (from mild debridement to amputation) may be required, especially when deep structures are involved (*2*).

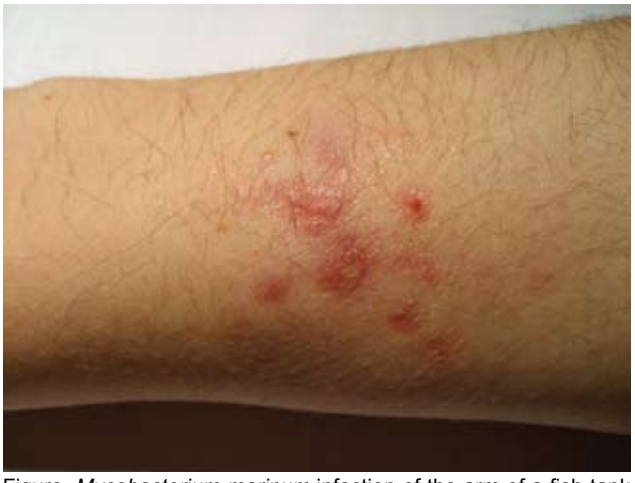

Figure. *Mycobacterium marinum* infection of the arm of a fish-tank worker.

M. ulcerans is the causative agent of Buruli ulcer, a disease reported in >30 countries, mainly in tropical and subtropical regions of Western and Central Africa but also in Central and South America, Southeast Asia, and the Western Pacific region (*26*). Currently limited knowledge is mainly the result of a low number of reported cases and inadequate surveillance. Recently, however, studies conducted in some areas in which *M. ulcerans* is highly endemic have reported infection rates higher than those for either tuberculosis or leprosy (*27*). Epidemiologic data on detection of *M. ulcerans* DNA in aquatic insects (e.g., or-

ders *Odonata* and *Coleoptera*) and snails, as well as in the biofilm of aquatic plants, strongly suggest that the organism is associated with exposure to surface water involved in environmental changes such as mining, deforestation, agriculture, and hydraulic installations and that it is likely to occupy a specific niche within aquatic environments from which it is transmitted to humans by an unknown mechanism (*28*). It is hypothesized that *M. ulcerans* reaches the human dermis through a cut or wound contaminated with water, soil, or vegetation. *M. ulcerans* is a unique species able to produce a potent, virulence-associated toxin called mycolactone, which prevents phagocytosis of live organisms and induces tissue destruction by its cytotoxic and immunosuppressive properties. Although all age groups can be affected, Buruli ulcer is more frequent among children <15 years of age; the lower limbs (which are involved 3.2× more often than the upper limbs) are the most frequently affected sites (*29*). The incubation period varies but is generally <3 months (*4*). Buruli ulcer usually starts as a single painless subcutaneous nodule or papule, which later moves to form an ulcer with undermined edges (*30*). Spontaneous healing usually takes 4–6 months and involves extensive scar formation, resulting in severe deformity with joint contracture, subluxation, atrophy, or distal lymph edema (*4*). Sometimes tissue destruction may be so extensive that amputation is unavoidable. In addition, dissemination to distant sites can occur, especially in younger patients (<15 years). Multiple lesions represent the most severe form of the disease; a high percentage of cases are osteomyelitis, often leading to amputation or even death (*29*). Disabilities are frequent, estimated for 25% to 58% of cases (*31*). Although in *M. ulcerans*–endemic areas, diagnosis and treatment are determined essentially by clinical appearances, laboratory methods are available. These methods consist of direct smear examination of specimens taken from the ulcer edge or from tissue (≈40% sensitivity); culture incubated at 29°C–33°C for 6–8 weeks (≈20%–60% sensitivity); histopathologic necrosis of subcutaneous tissues and dermal collagen accompanied by a scant nongranulomatous inflammatory reaction embedding acid-fast bacilli (≈90% sensitivity) (*30*); and PCR, a highly sensitive test that can produce results within 2 days but is still confined to reference and research laboratories (*32*). Case confirmation requires at least 2 positive results from the above diagnostic tests (*33*). A standardized method for susceptibility testing of *M. ulcerans* is not currently available (*25*). The main treatment for Buruli ulcer is surgery. To ensure complete removal of visibly affected tissue and to prevent recurrence, excision should include a wide margin, including healthy tissue. Although in early disease simple excision is usually curative, in advanced disease wide and traumatizing excision is needed, followed by skin grafting and long hospital stays. Although many antimicrobial agents have demonstrated excellent in vitro and in vivo activity against *M. ulcerans* clinical isolates, further studies are needed to assess whether experimental susceptibility data will correlate with clinical outcome (*33*). A recent report stated that rifampin plus streptomycin (1×/day for 4 weeks) and surgical excision inhibited the spread of infection and converted early lesions (nodules and plaques) from culture positive to culture negative (*34*). In edematous *M. ulcerans* disease, the most rapidly progressive form, rifampin and streptomycin have demonstrated a dramatically beneficial effect. Other treatments (e.g., topical phenytoin powder, topical nitrogen oxides, and dressing plus triple-drug therapy [rifampin, amikacin, and heparin]) are still being evaluated. *M. bovis* BCG vaccination appears to offer some short-term protection, especially against the most severe form of the disease (*33*).

Cutaneous MAC disease occurs by direct inoculation (trauma, surgery, injection) and is characterized by skin lesions such as ulceration, abscess with sinus formation, or erythematous plaque with a yellow crusted base. The lesions are indolent, with little or no lymph node reaction or systemic symptoms; some MAC skin infections resemble *Lupus vulgaris* infections. Diagnosis requires a high index of suspicion; a history of exposure to a potential source of infection may be suggestive. For an accurate diagnosis, histopathology, proper acid-fast bacilli identification, and susceptibility testing are needed. A combination of excision (or surgical debridement) and chemotherapy is usually required. Therapy is continued for 6–12 months and consists of at least 3 drugs, usually clarithromycin, rifampin, and ethambutol. Additional therapy with amikacin is sometimes included for 6 weeks (*2*).

Rapidly growing mycobacteria (RGM) are a complex group of environmental pigmented and nonpigmented mycobacteria. Their optimal incubation temperatures range from 25°C to 40°C, and they are characterized by a rapid (within 7 days) growth rate on subculture. Organisms responsible for disease in humans belong to the *M. fortuitum* group, the *M. chelonae/abscessus* group, and the *M. smegmatis* group; *M. abscessus*, *M. fortuitum*, and *M. chelonae* are the most common species involved in cutaneous and soft tissue infections. Able to survive in harsh conditions, these organisms produce biofilm in aquatic environments, mostly in piped water systems from which large clumps of mycobacteria are released into water and can be subsequently transmitted to humans (*35*,*36*). In addition, RGM are resistant to sterilizing agents (2% formaldehyde and glutaraldehyde), antiseptics (organomercurial compounds), and other common disinfectants (*37*). Clinical manifestations of RGM disease largely depend on the immunocompetence of the infected person. Cutaneous and soft tissue infections may appear as a single lesion in an immunocompetent person, usually after penetrating trauma or invasive

surgical procedure at the site of infection (*M. fortuitum* is the predominant causative agent), or they may appear as multiple or disseminated lesions, usually associated with immunosuppressive treatments (especially long-term treatment with steroids) or other immunosuppressive conditions or concurrent illnesses (*38*). For the latter, *M. chelonae* and *M. abscessus* are the predominant causative agents. Several reported infections that occurred after traumatic, cosmetic, or other medical procedures are summarized in Table 3. In a large outbreak of RGM infection related to pedicures, the median onset of signs and symptoms was 3 weeks, but for some, signs and symptoms were delayed as long as 4 months after exposure (*39*). Histopathologic examination of lesions showed suppurative granulomata and abscesses. Areas of necrosis were typically seen, but caseation was uncommon (*21*). Definitive diagnosis of clinically suspected RGM soft tissue disease can be made by culture of organisms from drainage material, aspiration fluid, or tissue biopsy sample. When NTM disease is suspected on the basis of clinical signs, with any patient history, laboratory staff should be alerted so they can use appropriate isolation protocols (*M. chelonae* and some strains of *M. abscessus* are relatively heat intolerant and can be recovered by primary isolation at 30°C). Identification of RGM at the species level is of utmost importance because treatment regimens and consequently clinical outcome are strongly species related. Proper identification involves molecular techniques (*22*) coupled with a few traditional biochemical and phenotypic tests (use of the carbohydrates citrate, mannitol, inositol, and sorbitol; tolerance to 5% NaCl; nitrate reduction; iron uptake; and 3-day arylsulfatase activity) (*35*). Susceptibility testing performed by the broth microdilution technique (*25*) is essential for choosing the most effective drug therapy and monitoring for the development of mutational drug resistance (*2*). Most experts recommend the use of specific antimicrobial drugs, given in combination to avoid the emergence of drug resistance. Treatment for the *M. fortuitum* group may include amikacin, cefoxitin, ciprofloxacin, the newer quinolones gatifloxacin and moxifloxacin, sulfonamides, and imipenem. Some degree of susceptibility to doxycycline and clarithromycin has been reported. *M. abscessus* strains are usually susceptible to amikacin, cefoxitin, imipenem, clarithromycin, and azithromycin; *M. chelonae* are usually susceptible to amikacin, imipenem, tobramycin, clarithromycin, and sometimes linezolid. Clarithromycin is generally the drug of choice for localized disease caused by *M. chelonae* and *M. abscessus* (*5*,*35*). The duration of therapy is usually 4 months for mild disease and 6 months for severe disease. Surgery is an important complementary tool for treating these infections, depending on disease severity and location.

Laboratory Diagnosis

Of all the currently described mycobacterial species, ≈60% have caused human diseases. For this reason, modern techniques for faster culture, identification, and drug susceptibility testing are urgently needed in mycobacteriology laboratories (*22*,*25*). In addition to the collection of high-quality specimens, timely diagnosis of NTM disease requires regular communication of clinical suspicion to the laboratory staff because optimal recover of some fastidious species requires additional tasks. Routine techniques include microscopy and culture; the latter should be performed by using both liquid and solid media incubated at

Table 3. Skin and soft tissue infections caused by rapidly growing mycobacteria

Type of infection or procedure	*Mycobacterium* spp.	Clinical findings
Posttraumatic wound infections	*M. fortuitum, M. chelonae, M. abscessus, M. wolinskyi, M. goodii, M. porcinum*	Subcutaneous abscesses, cellulitis
Pedicures	*M. fortuitum, M. chelonae, M. mageritense*	Furunculosis
Subcutaneous, intraarticular, or periarticular Injections	*M. chelonae, M. abscessus*	Subcutaneous abscesses, painful nodules, multiple sinus tracts, joint infections, fever, chills after injection
Acupuncture	*M. abscessus, M. chelonae, M. nonchromogenicum*	Erythematous papules, nodules, ulcerative lesions, abscesses, confluent plaques, draining sinus tracts with discharge, wrist tenosynovitis
Cardiac surgery	*M. peregrinum, M. fortuitum, M. fortuitum* (third biovariant) *complex, M. wolinskyi, M. goodii, M. abscessus*	Sternal wound infection, endocarditis
Cosmetic surgery or other surgical procedures: liposuction, liposculpture, face lift, breast lift (reduction augmentation), silicon injection	*M. fortuitum, M. chelonae, M. chelonae, M. porcinum, M. wolinskyi, M. goodii*	Erythema, tenderness, nodules, skin induration, subcutaneous or deep-tissue abscesses, fever, malaise, multiple abscesses along original suction tracts, draining sinus tracts, local pain, swelling
Nipple piercing	*M. fortuitum, M. abscessus*	Asymptomatic nodules, tender nodules
Implanted with prosthetic material	*M. abscessus, M. goodii*	Abscesses
Pacemaker placement	*M. fortuitum, M. abscessus, M. wolinskyi*	Abscesses
Peritoneal dialysis catheter	*M. abscessus*	Abscesses

different temperatures (*22*). Although optimal recovery for most clinically relevant mycobacteria is obtained at 35C°–37C°, some species (*M. hemophilum*, *M. marinum, M. ulcerans* and some species of RGM) require a lower incubation temperature to grow. For this reason, all clinical specimens that may harbor the above species (skin, synovial fluid, and bone) should be cultured at 28C°–30C° and at 35C°–37C°. Use of conventional biochemical and phenotypic tests for the identification of NTM is currently discouraged; more rapid and specific methods are favored, including high-performance liquid chromatography analysis of mycolic acids and commercial molecular assays. These may use either in-solution hybridization (Accuprobe, GenProbe Inc., San Diego, CA, USA) or solid-format reverse-hybridization assays (line probe assays) (*22*).Both techniques are specific, but the latter (in which amplification precedes hybridization) is more sensitive, enabling identification in the early stage of bacterial growth. Finally, gene (16S rDNA) sequencing is required for those species that cannot be identified by the above systems (*22*). Careful strategies should be recommended for using 16S rDNA sequence analysis databases because public databases may have wrong sequences and commercial ones tend to be underdeveloped and outdated (*23*,*40*).

Conclusion

Although NTM cause a broad spectrum of human disease, data on incidence of NTM infections are still lacking, mainly because of the absence of systematic epidemiologic studies, standard case definitions, and accurate mycobacterial identification. Furthermore, nonspecific clinical manifestations, lack of familiarity with these infections, and inadequate laboratory services make definitive diagnosis of NTM diseases often delayed or even impossible. Correlation of in vitro susceptibility testing with the clinical outcome, composition and duration of treatment regimens, and use of surgery or other therapeutic approaches are still undefined for most NTM species involved in human diseases. Laboratory research and multicenter controlled trials are needed to improve diagnosis and treatment of extrapulmonary NTM infections.

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References

1. Falkinham JO. Epidemiology of infection by nontuberculosis mycobacteria. Clin Microbiol Rev. 1996;9:177–215.
2. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial disease. Am J Respir Crit Care Med. 2007;175:367–416. DOI: 10.1164/rccm.200604-571ST
3. Marchevsky AM, Damsker B, Green S, Tepper S. The clinicopathological spectrum of nontuberculous mycobacterial osteoarticular infections. J Bone Joint Surg Am. 1985;67:925–9.
4. Dobos KM, Quinn FD, Ashford DA, Horsburgh CR, King CH. Emergence of a unique group of necrotizing mycobacterial diseases. Emerg Infect Dis. 1999;5:367–78.
5. De Groote MA, Huitt G. Infections due to rapidly growing mycobacteria. Clin Infect Dis. 2006;42:1756–63. DOI: 10.1086/504381
6. Lai KK, Stottmeier KD, Sherman IH, McCabe WR. Mycobacterial cervical lymphadenopathy. Relation of etiologic agent to age. JAMA. 1984;251:1286–8. DOI: 10.1001/jama.251.10.1286
7. Lindeboom JA, Prins JM, Bruijnesteijn van Coppenraet ES, Lindeboom R, Kuijper EJ. Cervicofacial lymphadenitis in children caused by *Mycobacterium haemophilum.* Clin Infect Dis. 2005;41:1569–75. DOI: 10.1086/497834
8. Tortoli E. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev. 2003;16:319–54. DOI: 10.1128/CMR.16.2.319-354.2003
9. Ellison E, Lapuerta P, Martin SE. Fine needle aspiration diagnosis of mycobacterial lymphadenitis. Sensitivity and predictive value in the United States. Acta Cytol. 1999;43:153–7.
10. Lindeboom JA, Kuijper EJ, Prins JM, Bruijnesteijn van Coppenraet ES, Lindeboom R. Tuberculin skin testing is useful in the screening for nontuberculous mycobacterial cervicofacial lymphadenitis in children. Clin Infect Dis. 2006;43:1547–51. DOI: 10.1086/509326
11. Lindeboom JA, Kuijper EJ, Prins JM, Bruijnesteijn van Coppenraet ES, Lindeboom R, Prins JM. Surgical excision versus antibiotic treatment for nontuberculous mycobacteria cervical lymphadenitis in children: a multicenter, randomized, controlled trial. Clin Infect Dis. 2007;44:1057–64. DOI: 10.1086/512675
12. Petitjean G, Fluckiger U, Schären S, Laifer G. Vertebral osteomyelitis caused by non-tuberculous mycobacteria. Clin Microbiol Infect. 2004;10:951–3. DOI: 10.1111/j.1469-0691.2004.00949.x
13. Elsayed S, Read R. *Mycobacterium haemophilum* osteomyelitis: case report and review of the literature. BMC Infect Dis. 2006;6:70. DOI: 10.1186/1471-2334-6-70
14. Zenone T, Boibieux A, Tigaud S, Fredenucci JF, Vincent V, Chidiac C, et al. Non-tuberculous mycobacterial tenosynovitis: a review. Scand J Infect Dis. 1999;31:221–8. DOI: 10.1080/00365549950163482
15. Theodorou DJ, Theodorou SJ, Kakitsubata Y, Sartoris DJ, Resnick D. Imaging characteristics and epidemiological features of atypical mycobacterial infections involving the musculoskeletal system. AJR Am J Roentgenol. 2001;176:341–9.
16. Casal M, Casal MM; Spanish Group of Mycobacteriology. Multicenter study of incidence of *Mycobacterium marinum* in humans in Spain. Int. J Tuberc Lung Dis. 2001;5:197–9.
17. Hess CL, Wolock BS, Murphy MS. *Mycobacterium marinum* infections of the upper extremity. Plast Reconstr Surg. 2005;115:55e–9e. DOI: 10.1097/01.PRS.0000153197.64808.B9
18. Edelstein H. *Mycobacterium marinum* skin infections. Report of 31 cases and review of the literature. Arch Intern Med. 1994;154:1359–64. DOI: 10.1001/archinte.154.12.1359
19. Aubry A, Chosidow O, Caumes E, Robert J, Cambau E. Sixty-three cases of *Mycobacterium marinum* infection: clinical features, treatment, and antibiotic susceptibility of causative isolates. Arch Intern Med. 2002;162:1746–52. DOI: 10.1001/archinte.162.15.1746

20. Ang P, Rattana-Apiromyakij N, Goh CL. Retrospective study of *Mycobacterium marinum* skin infections. Int J Dermatol. 2000;39:343–7. DOI: 10.1046/j.1365-4362.2000.00916.x
21. Bartralot R, Pujol RM, García-Patos V, Sitjas D, Martín-Casabona N, Coll P, et al. Cutaneous infections due to nontuberculous mycobacteria: histopathological review of 28 cases. Comparative study between lesions observed in immunosuppressed patients and normal hosts. J Cutan Pathol. 2000;27:124–9. DOI: 10.1034/j.1600-0560.2000.027003124.x
22. Clinical and Laboratory Standards Institute. Laboratory detection and identification of mycobacteria: approved guideline. CLSI document M48-A. Wayne (PA): The Institute; 2008.
23. Cloud JL, Neal H, Rosenberry R, Turenne CY, Jama M, Hillyard DR, et al. Identification of *Mycobacterium* spp. by using a commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries. J Clin Microbiol. 2002;40:400–6. DOI: 10.1128/JCM.40.2.400-406.2002
24. Bråbäck M, Riesbeck K, Forsgren A. Susceptibilities of *Mycobacterium marinum* to gatifloxacin, gemifloxacin, levofloxacin, linezolid, moxifloxacin, telithromycin, and quinupristin-dalfopristin (Synercid) compared to its susceptibilities to reference macrolides and quinolones. Antimicrob Agents Chemother. 2002;46:1114–6. DOI: 10.1128/AAC.46.4.1114-1116.2002
25. National Committee for Clinical Laboratory Standards. Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes. Approved standard M24-A. Wayne (PA): The Committee; 2003.
26. World Health Organization. Buruli ulcer disease. *Mycobacterium ulcerans* infection: an overview of reported cases globally. Wkly Epidemiol Rec. 2004;79:194–200.
27. Amofah G, Bonsu F, Tetteh C, Okrah J, Asamoa K. Buruli ulcer in Ghana: results of a national case search. Emerg Infect Dis. 2002;8:167–70.
28. Marsollier L, Severin T, Aubry J, Merritt RW, Saint Andre JP, Legras P, et al. Aquatic snails, passive hosts of *Mycobacterium ulcerans.* Appl Environ Microbiol. 2004;70:6296–8. DOI: 10.1128/AEM.70.10.6296-6298.2004
29. Debacker M, Aguiar J, Steunou C, Zinsou C, Meyers WM, Scott JT, et al. *Mycobacterium ulcerans* disease: role of age and gender in incidence and morbidity. Trop Med Int Health. 2004;9:1297–304. DOI: 10.1111/j.1365-3156.2004.01339.x
30. Guarner J, Bartlett J, Whitney EA, Raghunathan PL, Stienstra Y, Asamoa K, et al. Histopathologic features of *Mycobacterium ulcerans* infection. Emerg Infect Dis. 2003;9:651–6.
31. Ellen DE, Stienstra Y, Teelken MA, Dijkstra PU, van der Graaf WT, van der Werf TS. Assessment of functional limitations caused by *Mycobacterium ulcerans* infections: towards a Buruli ulcer functional limitation score. Trop Med Int Health. 2003;8:90–6. DOI: 10.1046/j.1365-3156.2003.00976.x
32. Ross BC, Marino L, Oppedisano F, Edwards R, Robins-Browne RM, Johnson PD. Development of a PCR assay for rapid diagnosis of *Mycobacterium ulcerans* infection. J Clin Microbiol. 1997;35:1696–700.
33. Wansbrough-Jones M, Phillips R. Buruli ulcer: emerging from obscurity. Lancet. 2006;367:1849–58. DOI: 10.1016/S0140-6736(06)68807-7
34. Etuaful S, Carbonnelle B, Grosset J, Lucas S, Horsfield C, Phillips R, et al. Efficacy of the combination rifampin-streptomycin in preventing growth of *Mycobacterium ulcerans* in early lesions of Buruli ulcer in humans. Antimicrob Agents Chemother. 2005;49:3182–6. DOI: 10.1128/AAC.49.8.3182-3186.2005
35. Brown-Elliott BA, Wallace RJ Jr. Clinical and taxonomic status of pathogenic nonpigmented late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev. 2002;15:716–46. DOI: 10.1128/CMR.15.4.716-746.2002
36. Hall-Stoodley L, Stoodley P. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol. 2005;13:7–10. DOI: 10.1016/j.tim.2004.11.004
37. Wallace RJ Jr, Brown BA, Griffith DE. Nosocomial outbreaks/pseudo-outbreaks caused by nontuberculous mycobacteria. Annu Rev Microbiol. 1998;52:453–90. DOI: 10.1146/annurev.micro.52.1.453
38. Uslan DZ, Kowalski TJ, Wengenack LW, Virk A, Wilson JW. Skin and soft tissue infections due to rapidly growing mycobacteria. Arch Dermatol. 2006;142:1287–92. DOI: 10.1001/archderm.142.10.1287
39. Winthrop KL, Abrams M, Yakrus M, Schwartz I, Ely J, Gillies D, et al. An outbreak of mycobacterial furunculosis associated with footbaths at a nail salon. N Engl J Med. 2002;346:1366–71. DOI: 10.1056/NEJMoa012643
40. Turenne CY, Tschetter L, Wolfe J, Kabani A. Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. J Clin Microbiol. 2001;39:3637–48. DOI: 10.1128/JCM.39.10.3638-3648.2001

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Etiology of Encephalitis in Australia, 1990–2007

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Encephalitis is a clinical syndrome commonly caused by emerging pathogens, which are not under surveillance in Australia. We reviewed rates of hospitalization for patients with encephalitis in Australia's most populous state, New South Wales, from January 1990 through December 2007. Encephalitis was the primary discharge diagnosis for 5,926 hospital admissions; average annual hospitalization rate was 5.2/100,000 population. The most commonly identified pathogen was herpes simplex virus (n = 763, 12.9%). *Toxoplasma* encephalitis and subacute sclerosing panencephalitis showed notable declines. The average annual encephalitis case-fatality rate (4.6%) and the proportion of patients hospitalized with encephalitis with no identified pathogen (69.8%, range 61.5%–78.7%) were stable during the study period. The nonnotifiable status of encephalitis in Australia and the high proportion of this disease with no known etiology may conceal emergence of novel pathogens. Unexplained encephalitis should be investigated, and encephalitis hospitalizations should be subject to statutory notification in Australia.

Many novel infectious diseases have been reported since 1940; most have been zoonoses originating from wildlife (*1*). Several novel zoonotic viruses, including Hendra virus, Australian bat lyssavirus, and Nipah virus, have resulted in encephalitic illness in humans (*2–4*). Australia has witnessed the emergence of several zoonotic and arboviral pathogens associated with encephalitis; these pathogens have been either novel pathogens or pathogens appearing in new geographic locations and include Hendra virus, Murray Valley encephalitis virus, Australian bat lyssavirus, and Kunjin and Japanese encephalitis viruses (*5–7*). The appearance of these emerging pathogens that can result in encephalitis raises questions about the etiology of encephalitis in the Australian population and about the adequacy of surveillance for novel pathogens.

Encephalitis is an inflammatory process in the brain parenchyma and is associated with clinical evidence of brain dysfunction (*8*). An infectious etiology of encephalitis is usually suspected in a patient with fever, headache, and signs of diffuse brain dysfunction, often with focal neurologic signs (*9*,*10*). Encephalitis generally results in a serious illness requiring hospitalization. Severe encephalitic illness can lead to death, and survivors frequently experience ongoing neurologic sequelae (*9*,*10*).

Presumably, the most common etiologies of encephalitis are infectious (*11*), and viral pathogens account for most diagnosed cases. In developed countries, the most commonly identified pathogens associated with acute encephalitis are the herpes viruses (*9*,*12–15*). Herpes simplex encephalitis is believed to account for 10%–20% of cases (*12*), but pathogen identification is not always possible (*16*).

Several countries have conducted large epidemiologic studies to assess the impact of disease caused by encephalitis and to determine its etiology. Davison et al. reviewed UK hospital records for a 9-year period (1989–1998) and found that the hospitalization rate for viral encephalitis in UK hospitals was 1.5 cases per 100,000 population; 60% of cases were recorded as unidentified viral infection (*17*). During the study period in the United Kingdom, 419 deaths were attributed to viral encephalitis (overall case-fatality rate 6.5/100 cases), but an etiologic organism was iden-

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DOI: 10.3201/eid1509.081540

tified for only 50% of these deaths (*17*). A smaller prospective study conducted in Finland for a 25-year period (1967–1991) found no etiology for encephalitis in 49% of 322 patients hospitalized with this illness (*18*).

Khetsuriani et al. analyzed national data for the United States over a similar period (1988–1997) to estimate the impact of both viral and nonviral encephalitis hospitalizations (*11*). This study found that the hospitalization rate for encephalitis in the United States was 7.3 per 100,000 population, and no specific etiology was identified in 59.5% of cases. During the study period, a case-fatality rate of 7.4 per 100 cases was recorded. For those persons admitted with encephalitis for which an etiology was identified, most specified a viral etiology (*11*). Also in the United States, the California Encephalitis Study, a large prospective study, found that 63% of hospitalized patients with encephalitis from 1998 through 2005 had encephalitis of unknown etiology, despite extensive laboratory testing (*19*). In another US study of persons whose deaths were associated with encephalitis, 81.5%–86.2% of the deaths resulted from encephalitis with an unknown etiology (*20*).

In Australia, encephalitis, as a syndrome, is not a notifiable disease, and data about trends or even clusters of this disease are not routinely collected. Only laboratory-confirmed encephalitis cases due to certain pathogens (e.g., Murray Valley encephalitis virus, Japanese encephalitis virus, or Australian bat lyssavirus) are notifiable to public health units; thus, the occurrence of encephalitis is not well documented.

A single, small study conducted in Australia's tropical Northern Territory found that 18 (53%) of 34 encephalitis patients admitted to the Royal Darwin Hospital during a 5-year period (1992–1996) had encephalitis with unexplained etiology (*21*). This level of unknown pathogen etiology suggests that Australia has a similar rate of pathogen identification as that reported in the overseas studies. However, the relative importance of different pathogens may differ because Australia has unique pathogens such as Murray Valley encephalitis virus, Hendra virus, and Australian bat lyssavirus) (*5*).

In this study, we examine the impact of hospitalizations due to encephalitis in New South Wales (NSW), Australia's most populous state. We also describe trends in pathogen identification in patients hospitalized with encephalitis over the 18-year period 1990–2007.

Methods

Data Sources

Data on hospital discharges, deaths, and population for NSW were obtained for 1990–2007 from the Health Outcomes and Information Statistical Toolkit, a collection of databases maintained by the Epidemiology and Surveillance Branch of the NSW Department of Health. The datasets used were from the Inpatient Statistics Collection library. Encephalitis-associated hospital stays were extracted for the period for patients for whom data were complete by using International Classification of Diseases, 9th revision, Clinical Modification codes (ICD-9-CM, Jan 1990–Jun 1998) and International Classification of Diseases, 10th revision, Australian Modification (ICD-10-AM, July 1998–Dec 2007).

Data for encephalitis-associated deaths in NSW were extracted from the Australian Bureau of Statistics death library by using ICD-9 (1990–1997) and ICD-10 (1998–2006) codes. Data for deaths occurring in 2007 were unavailable at the time of extraction. Population statistics from the Australian Bureau of Statistics were accessed through the Health Outcomes and Information Statistical Toolkit for the same period.

Definitions

An encephalitis-associated hospital stay was defined as a hospitalization for which the primary discharge diagnosis was an ICD-9-CM or ICD-10-AM code for acute encephalitis. Relevant ICD-9-CM and ICD-10-AM codes were obtained by searching the alphabetical list of ICD codes for all codes that include the term enceph, but excluding nonencephalitis conditions (e.g., anencephaly) and including rabies. Additionally, 2 ICD-9-CM codes used for encephalitis-related conditions before July 1998 did not include the prefix enceph-, unlike nomenclatures used in ICD-10-AM encephalitis codes. One of these ICD-9-CM codes was 49.8, defined as other non–arthropod-borne viral diseases of central nervous system –other specified non–arthropod-borne viral diseases of the central nervous system. The other code was 49.9, defined as other non–arthropod-borne viral diseases of central nervous system –unspecified non–arthropod-borne viral diseases of the central nervous system. The use of these codes was found by comparing data extracted for 1998 and 1999. For these 2 years, coding was performed in both ICD-9-CM and ICD-10-AM, and data were then compared to identify the additional codes.

The Table shows the ICD-9-CM and ICD-10-AM encephalitis-associated conditions with codes used for encephalitis hospitalizations during 1990–2007. These codes were further classified by investigators into encephalitis with known pathogens or unknown pathogens (Table).

Data Analysis and Ethics

Encephalitis-associated hospitalizations were analyzed by using SAS version 8.0 (SAS Institute, Inc., Cary, NC, USA) by etiologic category, year, age, gender, and hospital location. Hospitalization rates and death rates

Table. Encephalitis-associated conditions with ICD-9-CM and ICD-10-AM codes most frequently used for primary encephalitis discharge diagnoses, New South Wales, Australia, 1990–2007*

Primary discharge diagnosis ICD code (ICD-9; ICD-10)	No. hospitalizations (% of total)
All hospitalizations	5,926 (100)
Known pathogens	1,800 (30.6)
Herpes viral encephalitis (54.3; B00.4)	763 (12.9)
Varicella encephalitis (52.0; B01.1)	226 (3.8)
Toxoplasma meningoencephalitis (130.0; B58.2)	221(3.7)
Acute disseminated encephalitis (G04.0 in ICD-10)	136 (2.3)
Zoster encephalitis (B02.0 in ICD-10)	105 (1.8)
Subacute sclerosing panencephalitis (46.2; A81.1)	79 (1.3)
Other specified non–arthropod-borne viral diseases of the central nervous system (49.8; A85.8)	71 (1.2)
Enteroviral encephalitis (A85.0 in ICD-10)	49 (0.8)
Listerial meningitis and meningoencephalitis (A32.1 in ICD 10)	28 (0.5)
Encephalitis, myelitis, and encephalomyelitis –postinfectious encephalitis (323.6 in ICD-9)	18 (0.3)
Measles encephalitis (55.0 in ICD 9)	15 (0.3)
Encephalitis, myelitis, and encephalomyelitis –encephalitis in viral diseases classified elsewhere (323.0 in ICD 9)	10 (0.2)
Rubella with neurologic complications –encephalomyelitis due to rubella (56.01 in ICD-9)	8 (0.1)
Meningococcal infection –meningococcal encephalitis (36.1 in ICD-9)	7 (0.1)
Viral encephalitis transmitted by other and unspecified arthropods (64 in ICD-9)	7 (0.1)
Bacterial meningoencephalitis and meningomyelitis, not elsewhere classified (G04.2 in ICD-10)	7 (0.1)
Mumps –mumps encephalitis (72.2 in ICD-9)	6 (0.1)
Other known pathogen codes (24 codes)	44 (0.7)
Unknown pathogens	4,126 (69.6)
Unspecified non–arthropod-borne viral diseases of the central nervous system or unspecified viral encephalitis (49.9; A86)	2,218 (37.4)
Encephalitis, myelitis, and encephalomyelitis –unspecified cause of encephalitis (323.9; G04.9)	1,648 (28.8)
Encephalitis, myelitis, and encephalomyelitis –other cause of encephalitis (323.8; G04.8)	260 (4.4)

*ICD-9-CM, International Classification of Diseases, 9th Revision, Clinical Modification; ICD-10-AM, International Classification of Diseases, 10th Revision, Australian Modification.

were calculated by using annualized population estimates from the Australian Bureau of Statistics. Negative binomial regression was used to analyze trends for hospitalization rates of all encephalitis hospitalizations and for those caused by known and unknown pathogens. The regression was repeated with adjustment for age groups over time by using log of the population as an offset and age as a categorical covariate. Trends were represented as annual percentage changes in rates. Ethical clearance was given by Hunter New England Population Health and by the Australian National University Human Research Ethics Committee.

Results

Encephalitis Hospitalizations

From January 1990 through December 2007, encephalitis accounted for 5,926 hospitalizations in NSW. The average number of hospitalizations per year was 329 (range 281–393). The most frequently identified etiology of encephalitis was herpes simplex virus infection, which accounted for 763 admissions (12.9%) (Table). Varicella encephalitis (226 admissions or 3.8%) and *Toxoplasma* meningoencephalitis (221 admissions or 3.7%) were also common known etiologies. The etiology of 4,126 admissions (69.6% of total admissions) was unknown.

The average annual rate of encephalitis hospitalization was 5.2 per 100,000 population (range 4.2–6.7) (Figure 1). The annual rate for total encephalitis cases was higher for men (average rate 5.7/100,000) than for women (4.7/100,000).

The case-fatality rate for encephalitis during 1990–2006 was 4.6/100 cases. Figure 2 shows the average rate of hospitalization for patients with encephalitis by 10-year age groups during 1990–2007. The highest rates of admission occurred for age groups 0–9 years and >60 years.

Pathogen Identification

The rate of encephalitis hospitalizations declined for admissions of patients with encephalitis with both known and unknown etiologies (Figure 1). A negative binomial regression model showed a statistically significant decline of 1.4% per year ($p = 0.0003$, 95% confidence interval [CI] 0.7%–2.2%) for total encephalitis admissions during 1990–2007. This trend was similar for cases of encephalitis with unknown etiologies (decline of 1.4% per year, $p = 0.0002$, 95% CI 0.7%–2.2%) and for encephalitis cases with known etiologies, although the effect was not statistically signifi-

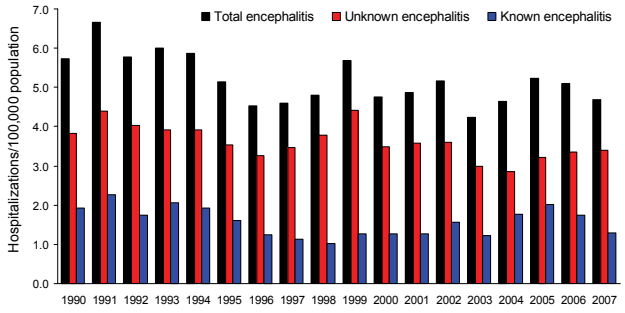

Figure 1. Encephalitis hospitalization rates by year and by known and unknown pathogen etiology, New South Wales, Australia, 1990–2007.

cant for pathogens with known etiologies (decline of 1.4% per year, p = 0.13, 95% CI 0.4%–3.2%,). This trend did not change when adjusted for age groups.

The proportion of cases with pathogens of known etiologies was higher for men than for women; the average hospitalization rate for men with encephalitis with a known etiology was 1.9/100,000 compared with 1.3/100,000 for women with encephalitis with a known etiology. In contrast, rates for patients with encephalitis with unknown etiologies were similar for men (3.8/100,000) and women (3.4/100,000). When the diagnosis of *Toxoplasma* encephalitis was excluded, the rate for hospitalizations for men with encephalitis with identified etiologies (1.5/100,000) was similar to the rate for women (1.3/100,000).

The proportion of hospitalizations with encephalitis with known etiology varied little among hospitals; a notable exception was a large Sydney hospital specializing in HIV-related medicine. For this hospital, the higher proportion of patient admissions with encephalitis with known etiology (50%) resulted from a high number of *Toxoplasma* encephalitis admissions.

Known Causes

Herpes encephalitis accounted for a relatively stable proportion of encephalitis hospitalizations during the study period (Figure 3). *Toxoplasma* encephalitis hospitalizations increased early in the study period and peaked in 1993 (Figure 4). Few toxoplasmosis hospitalizations occurred during the last 10 years of the study period. Subacute sclerosing panencephalitis (SSPE) was the only other diagnosis that decreased most years after 1994. These decreases appeared to contribute to the overall downward trend in hospitalizations with encephalitis with a known etiology, although this trend was not statistically significant; SSPE hospitalizations declined throughout the 1990s (Figure 5).

Discussion

Despite being uncommon (5.2/100,000 population annually), encephalitis is of public health importance because rapid response to cases may prevent additional transmitted cases of the underlying etiologies. Currently, nearly 70% of encephalitis cases have no identified etiology, so encephalitis is presently not amenable to public health prevention and control measures. This trend of encephalitis with unidentified etiology is seen elsewhere in developed countries and seems to occur even in the presence of extensive laboratory testing (*22*). Because of the number of emerging infectious diseases associated with encephalitis in Australia and elsewhere in recent times, surveillance should be enhanced.

The encephalitis hospitalization rate and case-fatality rate in NSW are lower than estimates from the United States (rate of 5.2 vs. 7.3/100,000/year, respectively; case-fatality rate of 4.6% vs. 7.4%, respectively); however, these differences may result in part from differences in study methods. The US study included hospitalizations for which encephalitis was the primary, secondary, or other discharge diagnosis, whereas our study included only those cases for which encephalitis was the primary discharge diagnosis. Similarly, a study in the United Kingdom found a hospitalization rate of 1.5 per 100,000 per year. The UK study included only cases of viral encephalitis and thus used a smaller number of ICD codes in the data collection than our study. This selection could account for the lower number of cases, although a true difference cannot be ruled out.

Our findings demonstrated a higher rate of encephalitis admissions that had no identified etiology (69.6%) in NSW than has been found in either the United States (59.5%) or the United Kingdom (60.0%). Although different methods of data extraction and different sample sizes may have contributed to these differences, a true difference in pathogens causing encephalitis could exist among these 3 countries. The high proportion of encephalitis cases with unknown etiology across all 3 countries emphasizes the limits of current diagnostic tools and/or the lack of systematic investigation of encephalitis cases. In Australia, the proportion of encephalitis deaths with no identified etiology increased from 47.0% during 1979–1992 to 57.2% during 1993–2006 (C. Huppatz, unpub. data). Although the datasets for hospital inpatients and for death registry data are not directly

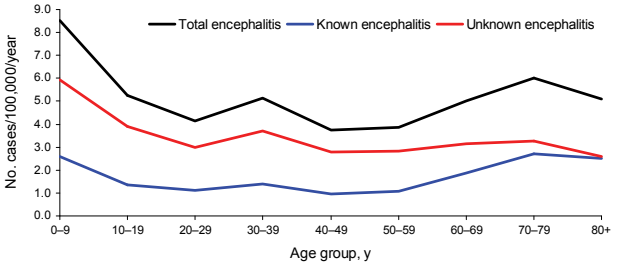

Figure 2. Average rates of encephalitis hospital admissions by 10-year age groups and by known and unknown pathogen etiology, New South Wales, Australia, 1990–2007.

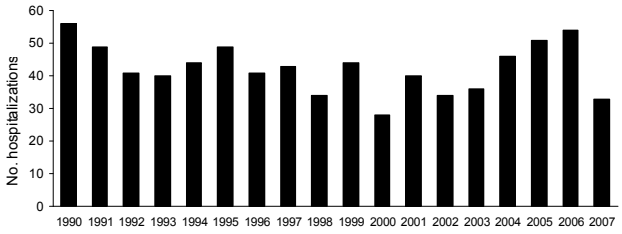

Figure 3. Herpes encephalitis hospitalizations by year, New South Wales, Australia, 1990–2007.

comparable, this relative increase in deaths from encephalitis with unknown etiology further emphasizes the need for enhanced routine surveillance.

Our data showed a higher rate of total encephalitis admissions for men (54.3%) compared with women (45.7%); this difference is largely due to a higher rate of toxoplasmosis encephalitis hospitalizations for men, a finding similarly observed in the US hospitalization data (*11*). *Toxoplasma* encephalitis is likely associated with HIV infection in the early 1990s.

The proportion of patients with encephalitis with unidentified etiology did not change during the study period, despite innovations in laboratory testing, particularly increased use of PCR testing after its described use for herpes encephalitis in 1990 (*23*). Interestingly, encephalitis with unknown etiology was not higher in rural areas, where lack of laboratory facilities and access to specialty units may constrain diagnosis. We found a similar rate of known to unknown etiologies for urban and rural hospitals, with the exception of 1 HIV referral center that had a high number of toxoplasmosis encephalitis admissions.

Our study showed that the most common known etiology was herpes virus (12.9%); encephalitis hospitalization rates changed little for patients with this virus during the study period, a finding consistent with the US and UK studies (*11*,*17*).

A decrease in encephalitis admissions due to *Toxoplasma* sp. was observed after a peak occurred for this pathogen in 1993. This decline in toxoplasmosis can be explained by the increasing use of prophylactic treatment for *Toxoplasma gondii* infection in people with HIV infection, a recommendation first widely published in Australia in 1994 (*24*).

The decrease in SSPE, a neurodegenerative disorder caused by the persistence of measles virus in the central nervous system (*25*), reflects the success of immunization in Australia. The median incubation period for the development of SSPE after measles infection is 6–8 years (*25*). Our data show a decline in SSPE admissions after this illness peaked in 1991. This decline most likely resulted from a national measles vaccination campaign in Australia in 1987 (*26*). The surprising increase of SSPE in 2005 can be partly explained by 3 admissions of 1 patient with SSPE. This patient's recurring hospitalizations were identified during a detailed review of encephalitis patients at the John Hunter Hospital, NSW, Australia (C. Huppatz, unpub. data). The decline in SSPE diagnoses builds on the growing evidence of the success of vaccination in decreasing illness and death from measles and its complications (*26*,*27*).

Several limitations are associated with use of hospital coding data to estimate the impact of disease. During the study period, the ICD coding system changed, and ICD-10 (1998–2007) contained several new codes not present in ICD-9 (1990–1997), including codes for zoster encephalitis, listeria meningoencephalitis, and enteroviral encephalitis. Before 1998, ICD-9 codes must have been designated to conditions associated with the new ICD-10 codes; however, which codes were used cannot definitely be determined. Fortunately, hospitalizations coded with the unknown ICD-9 codes account for a small proportion (<6%) of all encephalitis cases reported (Table).

In addition to limitations due to the ICD coding changes, differences may exist in diagnostic criteria for encephalitis used by clinicians and in inconsistencies among hospitals regarding coding practices and hospital admission criteria. Our data represent hospital admissions, rather than cases of encephalitis. Although this study criterion is useful for estimating the impact of disease as it relates to hospital service provision, case-fatality rates will be underestimated by the use of hospital admission data, rather than true cases, as some patients had multiple hospital admissions. The proportion of encephalitis cases with known and unknown etiologies remained constant, despite the ICD coding system change.

A previous study undertaken in the Northern Territory of Australia also found a high proportion (53%) of encephalitis cases with unknown etiologies (*21*). The Northern Territory has a distinctly different climate from that of NSW, and geographic variation exists in cases of encephalitis with known etiologies. For example, Murray Valley encephalitis and Kunjin encephalitis occur predominantly in the north. The relatively high proportion of encephalitis infections with unknown etiology appears consistent in these 2 different Australian regions.

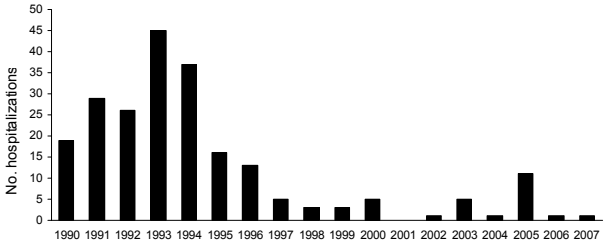

Figure 4. *Toxoplasma* encephalitis hospitalizations by year, New South Wales, Australia, 1990–2007.

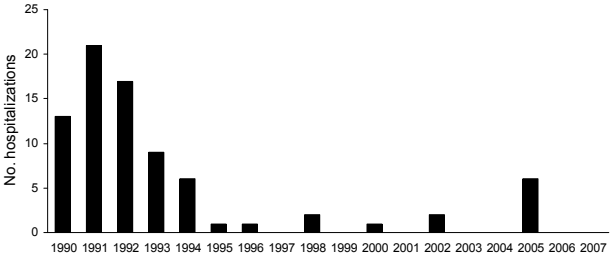

Figure 5. Subacute sclerosing panencephalitis hospitalizations by year, New South Wales, Australia, 1990–2007.

Conclusions

Acute encephalitis has heralded the emergence of multiple virulent pathogens (*28*), including West Nile virus, Hendra virus, Nipah virus, Murray Valley encephalitis virus, and Japanese encephalitis virus, all of which can cause severe illness and death (*2*,*4*,*29–31*). Although emerging infectious diseases may become apparent due to large outbreaks in humans, such as the Nipah virus outbreak in Malaysia (*4*), the diseases that emerged recently in Australia (e.g., Hendra virus infection and Australian bat lyssavirus infection) have been found in only a few persons (*2*,*32*). Consistent with US and UK studies (*11,17*), we found a high proportion (70%) of encephalitis cases with an unknown etiology in NSW. This finding highlights the need to monitor trends in encephalitis illness in Australia and to improve identification of the etiologies of encephalitis for detection of emerging infectious diseases. Based on our findings, we recommend that in Australia, surveillance be considered for encephalitis to assist in pathogen identification, alert authorities to outbreaks, and allow timely public health action.

Acknowledgments

We gratefully acknowledge the assistance of Megan Valentine and staff at the Epidemiology and Surveillance Branch of the New South Wales Department of Health; we also thank the Australian Government Department of Health and Ageing for funding the Master of Applied Epidemiology Program (C.H. and P.M.K.) and Australia's National Health and Medical Research Council for partially funding P.M.K.

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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. Barclay AJ, Paton DJ. Hendra (equine morbillivirus). Vet J. 2000;160:169–76. DOI: 10.1053/tvjl.2000.0508
3. Samaratunga H, Searle JW, Hudson N. Non-rabies lyssavirus human encephalitis from fruit bats: Australian bat lyssavirus (pteropid lyssavirus) infection. Neuropathol Appl Neurobiol. 1998;24:331–5. DOI: 10.1046/j.1365-2990.1998.00129.x
4. Bellini WJ, Harcourt BH, Bowden N, Rota PA. Nipah virus: an emergent paramyxovirus causing severe encephalitis in humans. J Neurovirol. 2005;11:481–7. DOI: 10.1080/13550280500187435
5. McCormack JG, Allworth AM. Emerging viral infections in Australia. Med J Aust. 2002;177:45–9.
6. Daley AJ, Dwyer DE. Emerging viral infections in Australia. J Paediatr Child Health. 2002;38:1–3. DOI: 10.1046/j.1440-1754.2002.00744.x
7. Mackenzie JS. Emerging zoonotic encephalitis viruses: lessons from Southeast Asia and Oceania. J Neurovirol. 2005;11:434–40. DOI: 10.1080/13550280591002487
8. Steiner I, Budka H, Chaudhuri A, Koskiniemi M, Sainio K, Salonen O, et al. Viral encephalitis: a review of diagnostic methods and guidelines for management. Eur J Neurol. 2005;12:331–43. DOI: 10.1111/j.1468-1331.2005.01126.x
9. Chaudhuri A, Kennedy PG. Diagnosis and treatment of viral encephalitis. Postgrad Med J. 2002;78:575–83. DOI: 10.1136/pmj.78.924.575
10. Whitley RJ, Gnann JW. Viral encephalitis: familiar infections and emerging pathogens. Lancet. 2002;359:507–13. DOI: 10.1016/S0140-6736(02)07681-X
11. Khetsuriani N, Holman RC, Anderson LJ. Burden of encephalitis-associated hospitalizations in the United States, 1988–1997. Clin Infect Dis. 2002;35:175–82. DOI: 10.1086/341301
12. Levitz RE. Herpes simplex encephalitis: a review. Heart Lung. 1998;27:209–12. DOI: 10.1016/S0147-9563(98)90009-7
13. Koskiniemi M, Rantalaiho T, Piiparinen H, von Bonsdorff CH, Farkkila M, Jarvinen A, et al. Infections of the central nervous system of suspected viral origin: a collaborative study from Finland. J Neurovirol. 2001;7:400–8. DOI: 10.1080/135502801753170255
14. Shoji H, Azuma K, Nishimura Y, Fujimoto H, Sugita Y, Eizuru Y. Acute viral encephalitis: the recent progress. Intern Med. 2002;41:420–8. DOI: 10.2169/internalmedicine.41.420
15. Schmutzhard E. Viral infections of the CNS with special emphasis on herpes simplex infections. J Neurol. 2001;248:469–77. DOI: 10.1007/s004150170155
16. Kennedy PG. Viral encephalitis: causes, differential diagnosis, and management. J Neurol Neurosurg Psychiatry. 2004;75(Suppl 1):i10–5. DOI: 10.1136/jnnp.2003.034280
17. Davison KL, Crowcroft NS, Ramsay ME, Brown DW, Andrews NJ. Viral encephalitis in England, 1989–1998: what did we miss? Emerg Infect Dis. 2003;9:234–40.
18. Rantalaiho T, Farkkila M, Vaheri A, Koskiniemi M. Acute encephalitis from 1967 to 1991. J Neurol Sci. 2001;184:169–77. DOI: 10.1016/S0022-510X(01)00441-5
19. Glaser CA, Honarmand S, Anderson LJ, Schnurr DP, Forghani B, Cossen CK, et al. Beyond viruses: clinical profiles and etiologies associated with encephalitis. Clin Infect Dis. 2006;43:1565–77. DOI: 10.1086/509330
20. Khetsuriani N, Holman RC, Lamonte-Fowlkes AC, Selik RM, Anderson LJ. Trends in encephalitis-associated deaths in the United States. Epidemiol Infect. 2007;135:583–91. DOI: 10.1017/S0950268806007163
21. Skull SA, Krause V, Dalton CB, Roberts LA. A retrospective search for lyssavirus in humans in the Northern Territory. Aust N Z J Public Health. 1999;23:305–8. DOI: 10.1111/j.1467-842X.1999.tb01261.x
22. Glaser CA, Gilliam S, Schnurr D, Forghani B, Honarmand S, Khetsuriani N, et al. In search of encephalitis etiologies: diagnostic challenges in the California Encephalitis Project, 1998–2000. Clin Infect Dis. 2003;36:731–42. DOI: 10.1086/367841

23. Rowley AH, Whitley RJ, Lakeman FD, Wolinsky SM. Rapid detection of herpes-simplex-virus DNA in cerebrospinal fluid of patients with herpes simplex encephalitis. Lancet. 1990;335:440–1. DOI: 10.1016/0140-6736(90)90667-T
24. Victorian Drug Usage Advisory Committee. Antibiotic guidelines. 8th ed. Melbourne, Victoria, Australia: Victorian Medical Postgraduate Foundation Therapeutics Committee; 1994.
25. Wong EH, Hui AC, Mok VC, Leung H, Chan RC, Wong KS, et al. A young man who kept falling over. Lancet. 2008;372:418. DOI: 10.1016/S0140-6736(08)61163-0
26. Turnbull FM, Burgess MA, McIntyre PB, Lambert SB, Gilbert GL, Gidding HF, et al. The Australian Measles Control Campaign, 1998. Bull World Health Organ. 2001;79:882–8.
27. Gidding HF. The impact of Australia's measles control programme over the past decade. Epidemiol Infect. 2005;133:99–105. DOI: 10.1017/S0950268804003073
28. Solomon T. Exotic and emerging viral encephalitides. Curr Opin Neurol. 2003;16:411–8. DOI: 10.1097/00019052-200306000-00023
29. Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ. West Nile virus. Lancet Infect Dis. 2002;2:519–29. DOI: 10.1016/S1473-3099(02)00368-7
30. Burrow JN, Whelan PI, Kilburn CJ, Fisher DA, Currie BJ, Smith DW. Australian encephalitis in the Northern Territory: clinical and epidemiological features, 1987–1996. Aust N Z J Med. 1998;28:590–6.
31. Mackenzie JS, Johansen CA, Ritchie SA, van den Hurk AF, Hall RA. Japanese encephalitis as an emerging virus: the emergence and spread of Japanese encephalitis virus in Australasia. Curr Top Microbiol Immunol. 2002;267:49–73.
32. Speare R, Skerratt L, Foster R, Berger L, Hooper P, Lunt R, et al. Australian bat lyssavirus infection in three fruit bats from north Queensland. Commun Dis Intell. 1997;21:117–20.

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Susceptibilities of Nonhuman Primates to Chronic Wasting Disease

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Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy, or prion disease, that affects deer, elk, and moose. Human susceptibility to CWD remains unproven despite likely exposure to CWD-infected cervids. We used 2 nonhuman primate species, cynomolgus macaques and squirrel monkeys, as human models for CWD susceptibility. CWD was inoculated into these 2 species by intracerebral and oral routes. After intracerebral inoculation of squirrel monkeys, 7 of 8 CWD isolates induced a clinical wasting syndrome within 33–53 months. The monkeys' brains showed spongiform encephalopathy and protease-resistant prion protein (PrPres) diagnostic of prion disease. After oral exposure, 2 squirrel monkeys had PrPres in brain, spleen, and lymph nodes at 69 months postinfection. In contrast, cynomolgus macaques have not shown evidence of clinical disease as of 70 months postinfection. Thus, these 2 species differed in susceptibility to CWD. Because humans are evolutionarily closer to macaques than to squirrel monkeys, they may also be resistant to CWD.

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are neurodegenerative diseases that affect many mammalian species. Some examples include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, Creutzfeldt-Jakob disease (CJD) in humans, and chronic wasting disease (CWD) in cervids. CWD was first found in captive deer in Colorado in 1967 (*1*) and was later identified in several US states and Canadian provinces (*2*). Epidemiologic evidence suggests that CWD continues to spread among cervid populations in North America (*3*), creating concern that CWD may cross species barriers to infect humans or domestic animals that may be eaten by humans. Thus, the host range of CWD and the level of protection provided by species barriers should be determined.

Substantial progress has been made in testing species barriers for CWD by using transgenic mice expressing species-specific prion protein (PrP), by direct infection into new species, or by in vitro conversion assays. The most sensitive method for testing susceptibility to TSE agents is intracerebral injection. Unfortunately, this route does not mimic most natural situations and only enables assessment of whether the possibility of transmission exists. Hamir et al. infected cattle and sheep with CWD by the intracerebral route and found protease-resistant PrP (PrPres) in 5 of 13 cattle and 2 of 8 sheep, which indicated that these ruminant species can propagate CWD (*4*,*5*). However, oral exposure in these hosts apparently does not cause disease (*2*).

CWD cross-species transmission to nonagricultural and laboratory animals has shown variable levels of susceptibility depending on the route of transmission. For example, ferrets were 100% susceptible to CWD by intracerebral infection but were not susceptible to oral infection (*6*,*7*). Mink were only 25% susceptible to CWD by intracerebral infection and were not susceptible to oral infection

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DOI: 10.3201/eid1509.090253

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(*8*). CWD has been successfully transmitted and adapted to laboratory rodents, including hamsters, transgenic mice expressing hamster PrP, and transgenic mice overexpressing mouse PrP (*9*,*10*). In contrast, transgenic mice expressing human PrP were not susceptible to CWD by intracerebral infection (*11*,*12*), a finding that provided evidence for a human species barrier against CWD infection. However, work started in 1980 and published in 2005 by Marsh et al. showed that 2 squirrel monkeys (*Saimiri sciureus*) infected by the intracerebral route with brain homogenate from a single CWD-affected mule deer became clinically sick at 31 and 34 months postinfection, and both were positive for PrPres (*13*). This evidence that at least 1 species of nonhuman primate was susceptible to CWD weakened the conclusion that humans may be protected from CWD by a species barrier.

We addressed 4 questions raised by the original observation that squirrel monkeys are susceptible to CWD (*13*). First, we compared intracerebral and oral routes of infection. This comparison was of interest because the oral route is likely to be an important natural route of disease transmission, and susceptibility is known to be lower by this route in most models. Second, we compared 2 species of nonhuman primates, cynomolgus macaques (*Macaca fascicularis*) and squirrel monkeys, each of which has previously shown susceptibility to various human prion diseases (*14–16*). However, humans are believed to be evolutionarily closer to cynomolgus macaques than to squirrel monkeys (*17*), and cynomolgus macaques may be a more accurate model for a human species barrier. Third, because only 1 CWD source was tested by Marsh et al. (*13*), we studied 8 different pools of CWD representing wild and captive cervids, including mule deer, white-tailed deer, and elk, from separate regions in the United States. Fourth, we tested the species tropism of CWD agent passaged in squirrel monkeys.

Materials and Methods

A description of the materials and methods used in this study follows. Additional details are available in the online Technical Appendix, available from www.cdc.gov/EID/content/15/9/1366-Techapp.pdf.

Animal Research

All monkeys and mice were housed at the Rocky Mountain Laboratories (Hamilton, MT, USA). Experimentation followed protocols approved by the National Institutes of Health Rocky Mountain Laboratories Animal Care and Use Committee.

CWD Pools for Infection of Primates

CWD-positive brain homogenates were provided by E.S.W. and M.W.M. Contents of each pool were as follows: MD-1, 6 free-ranging mule deer from Wyoming (*18*); MD-2, 4 captive mule deer from Colorado; MD-3, 28 captive mule deer from Wyoming and Colorado (*2*,*19*); WTD-1, 7 captive white-tailed deer from Wyoming and Colorado (*18,20*); WTD-2, 1 wild white-tailed deer from Wyoming; Elk-1, 2 free-ranging elk from Wyoming (*18*); Elk-2, 6 elk from a South Dakota game farm; and Elk-3, 10 captive elk from Wyoming and Colorado. Normal elk brain was a pool from 2 elk from Montana obtained from Lynn Creekmore of the US Department of Agriculture.

Inoculation of Monkeys

For intracerebral injections, squirrel monkeys received either 2 mg or 20 mg brain in a total volume of 200 μL, and cynomolgus macaques received 5 mg in a total volume of 500 μL. Oral doses of 200 mg brain/mL were given on 5 different days at 2–6 day intervals. Squirrel monkeys received 3-mL doses; most macaques received 4-mL doses. The inoculum was given to anesthetized animals through a rubber gastric tube.

Inoculation of Transgenic Mice

Brain homogenates diluted in phosphate buffered balanced solution containing 2% fetal bovine serum were inoculated intracerebrally into young adult mice. Volumes were 50 μL.

Generation of Transgenic Mice Expressing Human PrP

Mice expressing human PrP (tgRM and tg66) were generated by using a transgene, cosSHa.HumPrP, which was created by ligating the human PrP open reading frame into the cosSHa.Tet vector (*21*). The transgene was inoculated into eggs of FVBn–mouse PrP null mice in the laboratories of R.R. (tg66) and L.C. (tgRM). Each line of mice overexpressed human PrP as tested by Western blot with monoclonal antibody 3F4.

Analysis of Protease-sensitive PrP and PrPres by Immunoblot

Tissues were prepared by making a 20% (wt/vol) homogenate in 0.01 M Tris buffer, pH 7.4. Samples to be analyzed for protease-sensitive PrP (PrPsen) contained the following protease inhibitors: 10 μmol/L leupeptin, 1 μmol/L pepstatin A, and 1 μg/mL aprotinin. Samples were sonicated for 1 min and centrifuged at 5,000 rpm for 10 min. Supernatants were mixed 1:1 in 2× sample buffer and boiled for 3 min before electrophoresis.

Preparation of samples for PrPres analysis has been described (*18*). Removal of carbohydrate residues from PrPres was performed by digestion with peptide-N-glycosidase F (*22*).

After electrophoresis, proteins were transferred to Immobilon polyvinylidene difluoride–P membranes (Milli-

pore, Billerica, MA, USA), and PrP bands were detected with antibodies 3F4 (residues 109–112) (*23*), D13 (residues 96–106) (*24*) (InPro Biotechnology, Inc., South San Francisco, CA, USA), or L42 (residues 145–163) (r-Biopharm, Darmstadt, Germany) (*25*). Bands were detected by using enhanced chemiluminescence substrate (GE Healthcare, Piscataway, NJ, USA).

Histopathologic and Immunohistochemical Analyses

Routine formalin fixation, embedding, and tissue-sectioning protocols were followed. Tissues were stained with hematoxylin and eosin and analyzed for pathologic changes. Immunohistochemical staining was performed by using an automated Nexus stainer (Ventana, Tucson, AZ, USA). Anti-PrP antibodies D13 and 3F4 were used for PrPres immunostaining as described (*26*,*27*).

Sequencing

Primate genomic DNA was purified from whole blood, and PCR products were amplified by using PuRe Taq Ready-To-Go PCR beads (GE Healthcare). Two primers from the extreme outer ends of the open reading frame, including the previously published forward primer HM-1 (*28*) with mPrP-780R (5′-TCCCACTATCAGGAAGATGAGG-3′) or a combination of outer primers with internal primers mPrP-397F (5′-CCTTGGTGGCTACATGCTG-3′) and mPrP-416R (5′-CCAGCATGTAGCCACCAAG-3′), were used. Assembly comparisons were made against human, elk, mule deer, cynomolgus macaque, and squirrel monkey by using Sequencher version 4.6 (Gene Codes, Ann Arbor, MI, USA).

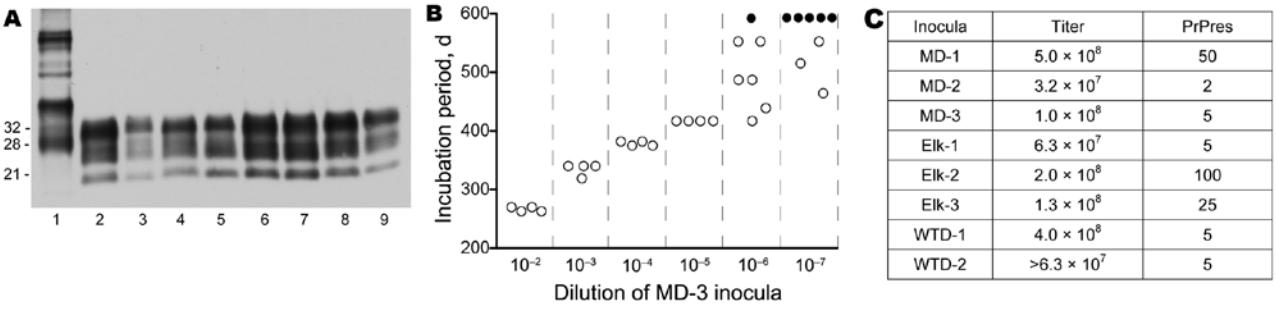
Results

Infectivity Levels in CWD Pools

When the 8 pools of CWD (representing both wild and captive deer and elk) used as inocula were analyzed by immunoblot, PrPres in the 8 pools showed similar electrophoretic mobilities and glycoform patterns (Figure 1, panel A), but PrPres levels differed when quantitatively compared (Figure 1, panel C). To measure the level of infectivity in these pools, we titered each pool in transgenic mice expressing deer PrP (line 33; tgDeerPrP) (*18*). A typical end-point dilution titration is shown in Figure 1, panel B. The 8 pools had 50% infectious dose (ID_{50}) titers ranging from 6.3×10^7 to 5.0×10^8 ID_{50}/g of brain homogenate (Figure 1, panel C). Comparison of titers with PrPres levels showed a partial correlation (Figure 1, panel C). For example, the CWD pool with the lowest infectivity titer (MD-2) was also the pool with the lowest PrPres level. However, for some pools, these tests showed discrepant values.

Intracerebral Infection of Squirrel Monkeys

To test susceptibility to CWD, we inoculated squirrel monkeys with each of the 8 CWD pools described above. Of 13 squirrel monkeys, 11 became symptomatic (33–53 mo postinfection [mpi]) (Table 1). The most consistent and reliable clinical finding was a severe wasting syn-



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Inocula	Titer	PrPres
MD-1	5.0×10^8	50
MD-2	3.2×10^7	2
MD-3	1.0×10^8	5
Elk-1	6.3×10^7	5
Elk-2	2.0×10^8	100
Elk-3	1.3×10^8	25
WTD-1	4.0×10^8	5
WTD-2	$>6.3 \times 10^7$	5

Figure 1. A) Western blot of chronic wasting disease (CWD) inocula showing protease-resistant prion protein (PrPres) in 8 CWD brain homogenate pools used for infecting nonhuman primates. Lane 1, 0.2-mg tissue equivalents of uninfected elk brain not treated with proteinase K; lanes 2–9, samples treated with proteinase K: lanes 2, 6, and 7, 0.12-mg tissue equivalents; lanes 3–5, 8, and 9, 0.67-mg tissue equivalents. PrPres was detected by using antibody L42 against PrP and enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA). To provide optimal exposure for viewing PrP in all lanes, blot was exposed to film for 20 min. In this exposure, lanes 2, 6, 7, and 8 were exposed beyond the linear range; this blot could not be used to quantify relative PrPres levels. Values on the left are in kDa. For more accurate quantitations of PrPres, other gels with different amounts loaded were exposed for multiple times (see panel C). B) Titration of MD-3 CWD inoculum. End-point infectivity titrations were calculated for each CWD inoculum by inoculating 50 μL of serial 10-fold dilutions of each brain homogenate into transgenic mice expressing deer PrP, starting with a 1% (10^{-2}) brain homogenate. Shown are data for an MD-3 inoculum. As the inoculum became more dilute, the incubation period (in days) and variability within a group increased. Each open circle represents 1 mouse in which clinical CWD developed. One mouse inoculated with a 10^{-6} dilution and 5 mice inoculated with a 10^{-7} dilution did not become sick after 625 days (solid circles). C) Infectivity titer and PrPres levels of each CWD pool. Titers are 50% infectious dose/g of brain homogenate. Relative level (%) of PrPres in each pool was measured by Western blot with a combination of serial dilutions and sequential exposure times in the linear response range for each sample. Data obtained from these comparisons are summarized in the PrPres column. All pools were compared with the pool with the highest PrPres signal (Elk-2), which was set at 100%.

Table 1. Results of squirrel monkey intracerebral inoculation with CWD agent*

Monkey no.†	PrP genotype‡	CWD inoculum	Titer inoculated§	Incubation period, mpi¶	Weight change, %
308	NT	MD-1	1.0×10^6	36	–8
633	A	MD-1	1.0×10^7	36	–42
334	B	MD-2	6.4×10^5	43	–38
393	B	MD-2	6.4×10^5	46	–28
640	A	MD-3	2.0×10^6	44	–35
365	NT	Elk-1	1.3×10^5	40	–43
643	A	Elk-1	1.3×10^6	53	–27
321	NT	Elk-2	4.0×10^5	35	–23
322	NT	Elk-3	2.6×10^5	33	–40
624	A	Elk-3	2.6×10^6	48	–37
399	A	WTD-1	8.0×10^6	50	–33
628	NT	WTD-1	8.0×10^6	NS (52)	0
310	A	WTD-2	1.3×10^5	NS (69)	+7
319	A	Normal elk		NS (69)	–8

*CWD, chronic wasting disease; PrP, prion protein; mpi, months postinfection; NT, not tested (sequenced); NS, no signs.
†In addition to the monkeys listed, 4 asymptomatic squirrel monkeys were euthanized at 10 mo after intracerebral inoculation with MD-1, MD-3, Elk-1, and WTD-1 to detect early accumulation of protease-resistant PrP (PrPres), but no PrPres was detected in brain by Western blot.
‡See Table 4 for a description of genotypes A, B, and C.
§Infectivity titers were determined by using endpoint dilution titer in transgenic deer expressing mouse PrP and are the 50% infectious dose/g of brain.
¶Incubation periods for monkeys with clinical wasting are indicated as mpi in parentheses. NS indicates that these monkeys did not show any clinical signs compatible with transmissible spongiform encephalopathy or wasting.

drome. Weight loss (average decrease of 33%) was most pronounced in the final few months of infection. Affected monkeys also had rough, poor-quality coats despite continuing to eat and drink. In the final 3–5 weeks, monkeys became weak and less active and spent most of their time hunched at the bottom of their cage. When the monkeys were encouraged to move, they did so slowly and deliberately. In the terminal stage of disease, a few had muscle tremors, excessive salivation, and mild ataxia. Fine, coordinated movement such as eating food was rarely affected. Monkeys were euthanized when terminal-stage weakness and wasting compromised their mobility and ability to eat and drink.

No clear correlation between incubation period and amount of agent inoculated was noted (Table 1). For example, 3 pairs of monkeys received the same inocula but in amounts that differed by 10-fold (Elk-1, Elk-3, and MD-1). Two pairs that received the lower dose became clinically sick first (Elk-1 and Elk-3). Both members of the third pair (MD-1) were euthanized after 36 months (Table 1). Two animals received the same dose of WTD-1 pool, yet to date, only 1 animal has become clinically sick. Animals that received the CWD pool with the lowest titer (MD-2) had incubation periods similar to those receiving much higher titered inocula (Table 1).

In all monkeys with clinical signs, CWD was confirmed by Western blot detection of PrPres in brain (Figure 2, panels A, B). The glycoform pattern of PrPres was similar for all affected monkeys inoculated with different CWD pools (Figure 2, panel B). Because PrPres deposition may also occur outside the central nervous system, we also tested peripheral lymphoid tissues. For 3 of 11 monkeys that had PrPres in brain, PrPres was also found in spleen and lymph nodes (Figure 2, panel C). In general, PrPres levels were much lower in lymphoid tissues than in brain and were often not detected by Western blot. All nonlymphatic tissues tested (cardiac muscle, skeletal muscle, duodenum, jejunum, ileum, colon, salivary gland, kidney, and lung) were negative for PrPres by immunoblot.

Tissues from squirrel monkeys euthanized after intracerebral injection with CWD (Table 1) were also examined by histopathologic analysis, including staining with hematoxylin and eosin and immunohistochemical detection of PrPres. All monkeys examined had spongiosis in the cerebral cortex, caudate, putamen, and thalamus (Figure 3, panel A). In addition, PrPres deposition was observed in many brain regions with large PrPres-positive plaques in the thalamus, cerebellum, and spinal cord (Figure 3, panels C, E, F) and in smaller plaques spread out in the gray matter of the internal capsule and white matter of the corpus callosum (Figure 3, panels G, H). The most abundant and consistent location for PrPres staining was found in the frontal cortex and in the fiber tracts of the claustrum (Figure 3, panel I). The adjacent caudate had severe spongiosis and astrocytosis but minimal PrPres (Figure 3, panel I). PrPres was also detected in lymph nodes and spleen, within follicles, in areas resembling follicular dendritic cells (Figure 3, panels K, M). Immunohistochemical analysis showed no PrPres in heart, kidney, adrenal gland, skeletal muscle, salivary gland, tongue, pancreas, white fat, and all regions of the gastrointestinal tract.

Oral Infection of Squirrel Monkeys

To test a more natural route of infection, we exposed squirrel monkeys orally to CWD. Of the 15 exposed squirrel monkeys, 1 (no. 345) was found dead in its cage at

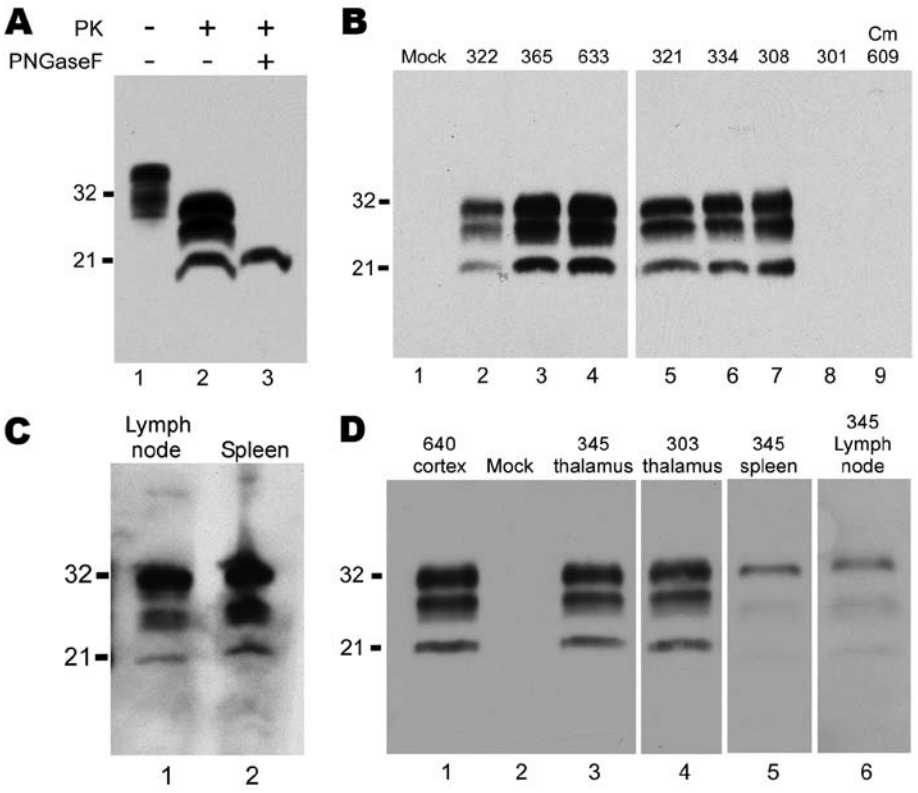

Figure 2. Western blots of squirrel monkey protease-resistant prion protein (PrPres). A) Brain homogenate from squirrel monkey 322, showing proteinase K (PK)–resistant PrPres. A downward shift of 7–9 kDa after PK digestion indicated a banding pattern typical of PrPres (lane 2). After deglycosylation with peptide-N-glycosidase F, 1 band of PrPres was present (lane 3). Lane 1, 0.5 mg of brain tissue equivalents; lane 2, 0.6 mg; lane 3, 0.4 mg. Blot was developed by using antibody 3F4 against PrP, enhanced chemiluminescence (ECL), and a 2-min exposure. B) Brains of monkeys screened for PrPres. All tissues were treated with PK, and lanes were loaded with 0.25 mg brain tissue equivalents, except for lane 5, which was loaded with 1.0 mg. All lanes contain samples of brain cortex except lanes 6 and 7, which contain thalamus. Blot was developed by using antibody 3F4, ECL, and a 30-min exposure. Lane 1, control. In lanes 2–7, PrP banding is similar among squirrel monkeys infected with different pools of chronic wasting disease (CWD) agent (Table 1). PrPres was not detected in brain of orally infected squirrel monkey 301 (lane 8) or in brain of an intracerebrally infected cynomolgus macaque (Cm) 609 (lane 9). C) Lymphatic tissues from squirrel monkey 365. For visualization of PrPres in lymph node and spleen, increased amounts of tissue were loaded, and a more sensitive detection system (femto detection; Thermo Scientific, Waltham, MA, USA) was used. Lane 1, lymph node, 0.7 mg tissue equivalents; lane 2, spleen, 1.1 mg tissue equivalents. Blot was developed by using antibody 3F4, femto-enhanced ECL, and a 1-min exposure. D) PK-treated brain and lymphatic tissues from orally infected squirrel monkeys 303 and 345. Lane 1, positive control no. 640; lane 2, negative control; lane 3, no. 345 thalamus; lane 4, no. 303 thalamus; lane 5, no. 345 spleen; lane 6, no. 345 mesenteric lymph node. Bands were visualized by using antibody 3F4 (residues 109–112) and ECL. Lanes 1–5, 10-min exposure; lane 6, overnight exposure; tissue equivalents loaded per lane: lanes 1–4, 0.25 mg; lane 5, 0.5 mg; lane 6, 1 mg. Values on the left of all blots are in kDa.

69 mpi; it had shown no neurologic signs or weakness. Western blot results indicated PrPres in brain, spleen, and lymph nodes (Figure 2, panel D). The level of PrPres in the brain of monkey 345 was comparable with that in end-stage intracerebrally inoculated monkeys; body weight at necropsy indicated a 33% decrease over the final 10 months. The high levels of PrPres and the severe wasting indicate that CWD infection could have been the cause of death. A second monkey, 303, was euthanized at 69 mpi because of suspicion of TSE after 2 weeks of progressive weakness, wasting, and eventual anorexia. PrPres analysis confirmed PrPres in brain (Figure 2, panel D), spleen, and lymph nodes. For monkeys 303 and 345, levels of PrPres in the lymph nodes and spleens were 10–100-fold lower than those in brain.

Two other orally infected monkeys were euthanized during the first 69 mpi (Table 2). Monkey 301 was euthanized at 39 mpi, after rapid onset of lethargy and anorexia that led to severe dehydration. Results of Western blot analysis for PrPres were negative in brain (Figure 1, panel B), spleen, lymph nodes, heart, skeletal muscle, duodenum, jejunum, ileum, colon, salivary gland, kidney, lung, and tonsil. However, immunohistochemical analysis detected PrPres in the spleen and 1 mesenteric lymph node from this monkey, indicating a low level of infection (Figure 3, panels J, K). Monkey 614 was euthanized at 44 mpi be-

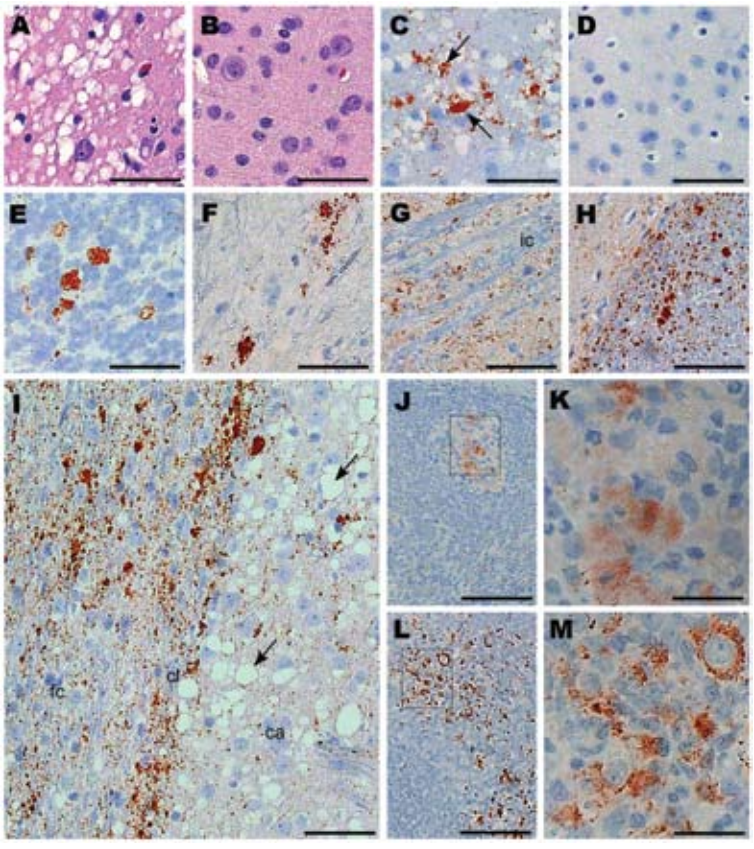

Figure 3. Immunohistochemical analysis of squirrel monkeys infected with chronic wasting disease (CWD) agent. Panels A, C, and E–M are from squirrel monkeys infected with CWD. Panels B and D are from an uninfected monkey showing no pathologic changes or positive staining for protease-resistant prion protein (PrPres). Panels A and B, cerebral cortex stained with hematoxylin and eosin; panels C and D, thalamus stained with antibody 3F4 against PrP (arrows); panels E and F, cerebellar granular cell layer and spinal cord, respectively, stained with antibody 3F4; panel G, gray matter within the internal capsule stained with antibody D13 against PrP; panel H, corpus callosum (right) stained with antibody D13 showing more intense staining than the adjacent cortex (left); panel I, frontal cortex (fc), claustrum (cl), and caudate (ca) stained with antibody 3F4 (abundant vacuoles in the putamen [arrows]); panels J–M, lymphatic tissue stained with antibody 3F4; panels J and K, PrPres staining in spleen of monkey 322; panels L and M, PrPres-positive mesenteric lymph node from orally infected monkey 301. Rectangles in panels J and L show areas enlarged in panels K and M, respectively. Antibodies D13 and 3F4 showed similar results for each monkey regarding the distribution, characteristics, and plaque size of PrPres. Scale bars: panels A–I, 50 μm; panel J, 100 μm; panels K and M, 25 μm; panel L, 250 μm.

cause it did not recover from anesthesia related to routine tuberculosis screening. Neither Western blot nor immunohistochemical analysis detected PrPres in brain, spleen, or lymph nodes of this monkey.

Infection of Cynomolgus Macaques

We inoculated cynomolgus macaques both orally and intracerebrally with 3 CWD inocula representing elk, mule deer, and white-tailed deer (Table 3). Of the cynomolgus macaques, 1 (no. 609) was euthanized at 48 mpi after it became aggressive. Brain (Figure 2, panel B), spinal cord, spleen, and lymph nodes were negative for PrPres by Western blot and immunohistochemical analysis. All remaining CWD-inoculated cynomolgus monkeys are currently (at 70 mpi) neurologically asymptomatic and have stable or increased body weights.

Sequences

Amino acid substitutions in PrP can alter susceptibility to TSE agents, including CWD (*18*,*29*,*30*). To determine whether the lack of susceptibility in several intracerebrally inoculated squirrel monkeys (Table 1) was caused by PrP gene polymorphisms, we sequenced the PrP genes from 23 squirrel monkeys. When compared with published squirrel monkey sequences (*28*,*31*), variation was seen at residue 164, in the number of octapeptide repeats, and at residue 19 of the signal peptide (Table 4). However, these genetic differences in PrP did not appear to account for the lack of susceptibility of monkey 310, which was genotype A, because this genotype was also found in 5 of the CWD-positive monkeys. Because we were not able to sequence PrP of monkey 628, we could not assess the role of PrP variation in the lack of disease.

Infectivity of CWD-infected Squirrel Monkey Tissues in PrP Transgenic Mice

To determine whether passage of CWD in squirrel monkeys altered the tropism of the infectious agent, we inoculated tgDeerPrP mice and tg mice expressing human PrP (lines 66 and RM) intracerebrally with tissue homogenates from 3 CWD-positive squirrel monkeys (nos. 322, 308, and 301) with PrPres and from an intracerebrally inoculated cynomolgus macaque (no. 609). Clinical disease did not develop in any tgDeerPrP mice during 600–700 days (Table 5). The lack of transmission to tgDeerPrP, tg66, or tgRM mice from the 3 squirrel monkeys with detectable CWD PrPres indicated that either the infectivity levels were low in these squirrel monkeys or that the original cervid species tropism was altered by the passage in squirrel monkeys. Similarly, the lack of transmission to tg mice expressing human PrP implied that passage through squirrel monkeys did not facilitate adaptation to an agent with increased tropism for humans.

Table 2. Results of squirrel monkey oral inoculation with CWD agent*

Monkey no.	PrP genotype†	CWD inoculum	Titer inoculated‡	Incubation period, mpi§	Weight change, %
303¶	NT	MD-1	1.5×10^9	69	–25
360	A	MD-1	1.5×10^9	NS (69)	+6
588	C	MD-3	9.6×10^7	NS (52)	+5
629	B	MD-3	9.6×10^7	NS (52)	0
631	A	Elk-1	1.9×10^8	NS (52)	0
335	NT	Elk-2	6.0×10^8	NS (69)	–5
656	B	Elk-2	6.0×10^8	NS (52)	–5
614#	A	Elk-2	6.0×10^8	44	–10
317	C	Elk-3	3.9×10^8	NS (69)	0
301**	NT	Elk-3	3.9×10^8	39	–14
307	A	WTD-1	1.2×10^9	NS (69)	+8
345††	A	WTD-1	1.2×10^9	69	–33
626	NT	WTD-2	1.9×10^8	NS (52)	+11
641	B	WTD-2	1.9×10^8	NS (52)	0
325	NT	WTD-2	1.9×10^8	NS (69)	–8
655	A	Buffer control		NS (52)	–6
314	NT	Normal elk		NS (69)	+7

*CWD, chronic wasting disease; PrP, prion protein; mpi, months postinfection; NT, not tested (sequenced); NS, no signs.
†See Table 4 for a description of genotypes A, B, and C.
‡Infectivity titers were determined by using endpoint dilution titer in transgenic deer expressing mouse PrP and are listed as the 50% infectious dose/g of brain administered over 5 d at 2–6-d intervals.
§Incubation periods for monkeys with clinical wasting are indicated as mpi in parentheses. NS indicates that these monkeys did not show any clinical signs compatible with transmissible spongiform encephalopathy (TSE) or wasting.
¶Monkey 303 was euthanized at 69 mpi because of signs of wasting, weakness, and anorexia.
#Monkey 614 was euthanized at 44 mpi because of complications arising from anesthesia for routine tuberculosis testing. No signs of TSE were observed before the complications, and Western blot and immunohistochemical results showed that this monkey was negative for protease-resistant PrP (PrPres).
**Monkey 301 was euthanized at 39 mpi after a brief illness with signs of lethargy, anorexia, and dehydration. PrPres was detected in peripheral lymphoid tissues but not in brain.
††Monkey 345 was found dead at 69 mpi. Brain, spleen, and lymph nodes were positive for PrPres by Western blot.

Discussion

As new CWD foci continue to emerge among cervid populations, the risk for CWD transmission to humans needs to be assessed. We used 2 monkey species and 2 routes of inoculation to test the susceptibility of primates to 8 different pools of CWD. To date, we have verified CWD in 11 of 13 intracerebrally inoculated squirrel monkeys; average incubation period was 41 months (range 33–53 months). Using a single CWD pool, Marsh et al. noted infection in 2 of 2 squirrel monkeys 31–34 months after intracerebral inoculation (*13*). Intracerebral inoculation of squirrel monkeys with other TSE agents, including agents of kuru, variant CJD, sporadic CJD, and sheep scrapie, had incubation periods of ≈24 months and attack rates of ≈100% (*14*,*15*,*32*). The extended incubation periods and lower attack rates for our squirrel monkeys may result from a partial species barrier to CWD.

The signs of wasting syndrome in CWD-infected monkeys were similar to those of CWD infection in cervids, in which loss of body condition is nearly always a major component of infection and neurologic deficits vary (*2*). The correlation of clinical signs between CWD in cervids and squirrel monkeys suggests that CWD might affect a common brain region in each species. We observed PrPres deposition in squirrel monkeys primarily in the frontal lobe of the cerebral cortex, claustrum, putamen, and thalamus. Cervids typically have the most abundant and predictable PrPres in the dorsal motor vagus nucleus (obex), olfactory cortex, and diencephalon (including thalamus, hypothalamus, metathalamus, and epithalamus) (*2*,*33*). A plausible

Table 3. Cynomolgus macaques infected with CWD agent*

No. monkeys	CWD inoculum	Route of inoculation	Titer inoculated†	Current mpi
1 (no. 609)‡	MD-1	Intracerebral	2.5×10^6	NA
1	MD-1	Intracerebral	2.5×10^6	70
3	MD-1	Oral	2.0×10^9	70
2	Elk-1	Intracerebral	3.2×10^5	70
3	Elk-1	Oral	2.5×10^8	70
2	WTD-1	Intracerebral	1.0×10^6	70
3	WTD-1	Oral	1.2×10^9	70

*CWD, chronic wasting disease; mpi, months postinfection; NA, not available.
†Infectivity titers were determined by using endpoint dilution titer in transgenic deer expressing mouse prion protein and are listed as the total 50% infectious dose/g of brain.
‡Monkey 609 was euthanized at 48 mpi, and no protease-resistant prion protein was detected by Western blot or immunohistochemical analysis.

Table 4. PrP sequence variability in squirrel monkeys*

Genotype†	No. monkeys	PrP gene variations‡		
		Residue 19	No. octapeptide repeats	Residue 164
A	16	Leu/Leu	5/5	Lys/Lys
B	5	ND	4/5	Lys/Lys
C	2	Val/Leu	5/5	Lys/Lys
Schätzl	1	Leu/Leu	5/5	Arg/Arg
Schneider	1	ND	4/4	Arg/Arg

*PrP, prion protein; ND, not determined.
†The PrP genes of 23 monkeys were sequenced, and 3 genotypes were found. For easy reference to Tables 1 and 2, they are designated types A, B, and C. Previous squirrel monkey PrP sequences were identified by Schätzl et al. (*28*) and Schneider et al. (*31*).
‡Compared with published sequences, PrP genotype variations were seen only at residue 19 (in the signal peptide), residue 164, and in the number of octapeptide repeats.

hypothesis could be that disruption of regions within the hypothalamus and thalamus leads to a metabolic imbalance, resulting in a severe wasting syndrome.

We did not observe a strong correlation between infectivity titer inoculated and attack incidence or incubation period (Table 1). One potential explanation is that the variation in speed of disease progression might not be relevant given the low number of animals in each group. A second possibility is that our squirrel monkeys varied at PrP alleles that may affect CWD susceptibility. However, analysis of 23 squirrel monkeys showed no PrP sequence differences correlating with susceptibility to CWD (Tables 1, 2, 4). A third possibility is that genes other than the gene for PrP might influence CWD susceptibility.

For humans, eating infected or contaminated tissue is a likely route of CWD exposure. In other animal models, oral transmission of TSE is generally 1,000-fold less effective than direct intracerebral challenge and results in longer incubation periods and lower efficiency of disease transmission. In our oral transmission experiments, we found evidence of CWD infection in 3 monkeys; 2 at 69 mpi had abundant PrPres in brain and lower levels in spleen and lymph nodes, and 1 euthanized at 39 mpi had PrPres in lymphatic tissues only. Thus, transmission seems to be slower by the oral route than by the intracerebral route, and other orally infected monkeys may be affected in the future.

Cynomolgus macaques are evolutionarily closer to humans than are squirrel monkeys (*17*). At nearly 6 years postinoculation, no macaques have shown clinical signs of CWD. Intracerebral inoculation of cynomolgus macaques with BSE causes disease in 3 years; human variant CJD requires 2–3 years, and human sporadic CJD requires 5 years (*16*,*34*). However, oral inoculation of cynomolgus macaques with BSE agent required a minimum of 5 years before clinical disease was observed (*35*). There-

Table 5. Infectivity of CWD agent from cervids, squirrel monkeys, and cynomolgus macaques in transgenic mice expressing deer PrP or human PrP*

Donor†	Original inoculum	Donor PrPres‡	TSE disease incidence§		
			tg33 (deer)	tg66 (human)	tgRM (human)
SM 322¶	Elk-3	+	0/8	NT	0/6
SM 308¶	MD-1	+	0/7	0/8	0/8
SM 301	Elk-3	±	0/6	NT	NT
SM 320	Uninfected	–	0/7	NT	NT
CM 609	MD-1	–	0/8	NT	NT
Elk-3	NA	+	6/6 (301 ± 11)	NT	NT
MD-1	NA	+	7/7 (323 ± 15)	NT	NT
sCJD (97–008)	NA	+	NT	NT	4/6 (170 ± 3)
sCJD (99–009)	NA	+	NT	NT	5/5 (194 ± 20)
sCJD (RR)	NA	+	NT	8/8 (163 ± 1)	NT
sCJD (PLG)	NA	+	NT	4/4 (163 ± 6)	NT

*CWD, chronic wasting disease; PrP, prion protein; TSE, transmissible spongiform encephalopathy; NT, not tested; sCJD, sporadic Creutzfeldt-Jakob disease.
†Each donor monkey inoculum was prepared as a 1% brain homogenate from the indicated monkeys. SM, squirrel monkey; CM, cynomolgus macaque. Elk and mule deer CWD inocula were described in Materials and Methods. Human sCJD inocula are brain homogenates from World Health Organization CJD reference materials. No. 99–009 is sCJD M/M type I, and no. 97–008 is sCJD M/M type II. The RR sample was from a patient with sCJD of unknown PrP genotype. The PLG sample (M/M type I) was from a patient with sCJD. In all cases except sCJD (RR), 50 μL was inoculated intracerebrally into recipient mice; for sCJD (RR), 30 μL was inoculated.
‡Based on Western blot or immunohistochemical analysis of brain for all except monkey 301, in which protease-resistant PrP (PrPres) was detected in spleen but not brain.
§Number of recipient mice with clinical transmissible spongiform encephalopathy confirmed by detection of brain PrPres is the numerator, and total number of mice inoculated is the denominator. Mean ± SEM incubation period for time to clinical disease is provided in days. Tg33 mice express deer PrP, and tg66 and tgRM mice express human PrP.
¶In addition to brain homogenate, we also inoculated tg33 mice with homogenates of spleen, lymph nodes, heart, muscle, and plasma from squirrel monkeys 322, 308, and 321, but disease did not develop during >600 d observation.

fore, we cannot rule out CWD transmission to cynomolgus macaques.

The PrP gene sequence can influence cross-species transmission of prion disease. Therefore, we compared squirrel monkey and cynomolgus macaque PrP gene sequences to look for differences that might account for different susceptibilities of these monkeys to CWD. In the PrP gene excluding the signal peptide, deer differed from squirrel monkeys at 17 residues and from cynomolgus macaques at 16 residues, but 14 of these differing residues were identical in squirrel monkeys and macaques (Figure 4). Therefore, there are only 2 residues in cynomolgus macaques (100 and 108) and 3 residues in squirrel monkeys (56, 159 and 182) at which these monkeys differ from deer and also from each other. These residues might play a role in susceptibility differences seen in our study.

Human exposure to CWD-infected cervids in past decades is likely. The highest levels of prion infectivity are present in the central nervous system and lymphatic tissues of CWD-infected cervids; contamination of knives, saws, and muscles with these tissues can easy occur when processing game. Despite the likelihood of exposures, epidemiologic studies of humans living in CWD-endemic areas of Colorado and Wyoming during 1979–2001 have not shown any increases in human TSE cases (*36*,*37*). Ongoing studies by the Colorado Department of Public Health and Environmental Human Prion Disease Surveillance Program, in conjunction with the University of Colorado School of Medicine, have also concluded that no convincing cases of CWD transmission to humans have been detected in Colorado (*38*). However, if CWD in humans appears like a wasting syndrome similar to that observed in the squirrel monkeys in our study, affected persons might receive a diagnosis of a metabolic disorder and never be tested for TSE. Fortunately, additional laboratory data are consistent with the epidemiologic data, and these results support the conclusion that a species barrier protects humans from CWD infection (*11–13*,*20*,*36*,*37*).

Acknowledgments

We thank John Portis, Byron Caughey, and Kristin McNally for critical review of the manuscript; Lori Lubke and Rebecca Rosenke for assistance with histologic analysis; Rocky Rivera and Ed Schreckendgust for animal husbandry; Anita Mora and Gary Hettrick for graphics assistance; Serguei Soukharev for assistance in creating transgenic mice expressing human PrP; Robert Rohwer for tg66 mice and sporadic CJD inocula; Terry Kreeger, Jean Jewell, and Lynn Creekmore for CWD-positive and CWD-negative cervid tissues; and Pier-Luigi Gambetti for a sample from a patient with sporadic CJD.

This study was supported by the National Institute of Allergy and Infectious Diseases, Division of Intramural Research.

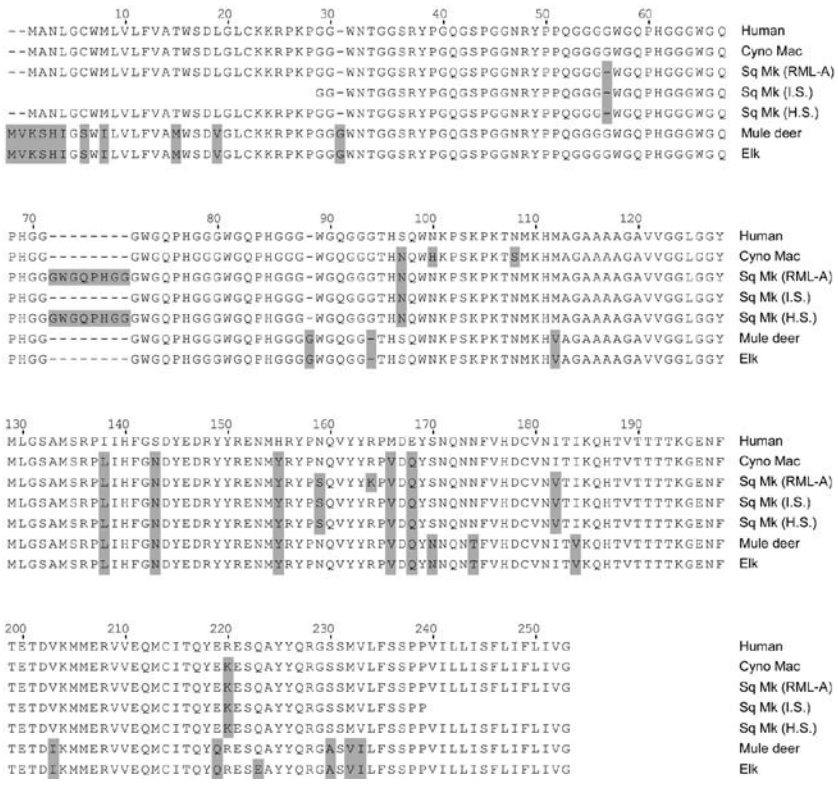

Figure 4. Comparison of prion protein sequences from various species. The following species are shown, and GenBank accession numbers are given when available: human (M13899), cynomolgus macaque (Cyno Mac) (U08298), squirrel monkey (Sq Mk) (genotype RML-A, see Table 4), squirrel monkey from Schneider et al. (*31*) (AY765385), squirrel monkey from Schätzl et al. (*28*) (U08310), mule deer (AY330343), and elk (AF156183). Numbering is based on the human sequence. Gray boxes indicate residues different from human residues. Alignment of the sequences was conducted with MegAlign software (DNAstar/Lasergene, Madison, WI, USA).

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References

1. Williams ES, Young S. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. J Wildl Dis. 1980;16:89–98.
2. Williams ES. Chronic wasting disease. Vet Pathol. 2005;42:530–49. DOI: 10.1354/vp.42-5-530
3. Miller MW, Williams ES. Chronic wasting disease of cervids. Curr Top Microbiol Immunol. 2004;284:193–214.
4. Hamir AN, Kunkle RA, Cutlip RC, Miller JM, O'Rourke KI, Williams ES, et al. Experimental transmission of chronic wasting disease agent from mule deer to cattle by the intracerebral route. J Vet Diagn Invest. 2005;17:276–81.
5. Hamir AN, Kunkle RA, Cutlip RC, Miller JM, Williams ES, Richt JA. Transmission of chronic wasting disease of mule deer to Suffolk sheep following intracerebral inoculation. J Vet Diagn Invest. 2006;18:558–65.
6. Sigurdson CJ, Mathiason CK, Perrott MR, Eliason GA, Spraker TR, Glatzel M, et al. Experimental chronic wasting disease (CWD) in the ferret. J Comp Pathol. 2008;138:189–96. DOI: 10.1016/j.jcpa.2008.01.004
7. Bartz JC, Marsh RF, McKenzie DI, Aiken JM. The host range of chronic wasting disease is altered on passage in ferrets. Virology. 1998;251:297–301. DOI: 10.1006/viro.1998.9427
8. Harrington RD, Baszler TV, O'Rourke KI, Schneider DA, Spraker TR, Liggitt HD, et al. A species barrier limits transmission of chronic wasting disease to mink (*Mustela vison*). J Gen Virol. 2008;89:1086–96. DOI: 10.1099/vir.0.83422-0
9. Raymond GJ, Raymond LD, Meade-White KD, Hughson AG, Favara C, Gardner D, et al. Transmission and adaptation of chronic wasting disease to hamsters and transgenic mice: evidence for strains. J Virol. 2007;81:4305–14. DOI: 10.1128/JVI.02474-06
10. Sigurdson CJ, Manco G, Schwarz P, Liberski P, Hoover EA, Hornemann S, et al. Strain fidelity of chronic wasting disease upon murine adaptation. J Virol. 2006;80:12303–11. DOI: 10.1128/JVI.01120-06
11. Kong Q, Huang S, Zou W, Vanegas D, Wang M, Wu D, et al. Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models. J Neurosci. 2005;25:7944–9. DOI: 10.1523/JNEUROSCI.2467-05.2005
12. Tamguney G, Giles K, Bouzamondo-Bernstein E, Bosque PJ, Miller MW, Safar J, et al. Transmission of elk and deer prions to transgenic mice. J Virol. 2006;80:9104–14. DOI: 10.1128/JVI.00098-06
13. Marsh RF, Kincaid AE, Bessen RA, Bartz JC. Interspecies transmission of chronic wasting disease prions to squirrel monkeys (*Saimiri sciureus*). J Virol. 2005;79:13794–6. DOI: 10.1128/JVI.79.21.13794-13796.2005
14. Brown P, Gibbs CJ Jr, Rodgers-Johnson P, Asher DM, Sulima MP, Bacote A, et al. Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease. Ann Neurol. 1994;35:513–29. DOI: 10.1002/ana.410350504
15. Williams L, Brown P, Ironside J, Gibson S, Will R, Ritchie D, et al. Clinical, neuropathologial and immunohistochemical features of sporadic and variant forms of Creutzfeldt-Jakob disease in the squirrel monkey (*Saimiri scuireus*). J Gen Virol. 2007;88:688–95. DOI: 10.1099/vir.0.81957-0
16. Lasmezas CI, Deslys JP, Demaimay R, Adjou KT, Lamoury F, Dormont D, et al. BSE transmission to macaques. Nature. 1996;381:743–4. DOI: 10.1038/381743a0
17. Hayasaka K, Gojobori T, Horai S. Molecular phylogeny and evolution of primate mitochondrial DNA. Mol Biol Evol. 1988;5:626–44.
18. Meade-White K, Race B, Trifilo M, Bossers A, Favara C, Lacasse R, et al. Resistance to chronic wasting disease in transgenic mice expressing a naturally occurring allelic variant of deer prion protein. J Virol. 2007;81:4533–9. DOI: 10.1128/JVI.02762-06
19. Sigurdson CJ, Williams ES, Miller MW, Spraker TR, O'Rourke KI, Hoover EA. Oral transmission and early lymphoid trophism of chronic wasting disease PrPres in mule deer fawns (*Odocoileus hemionus*). J Gen Virol. 1999;80:2757–64.
20. Raymond GJ, Bossers A, Raymond LD, O'Rourke KI, McHolland LE, Bryant PK III, et al. Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. EMBO J. 2000;19:4425–30. DOI: 10.1093/emboj/19.17.4425
21. Scott MR, Kohler R, Foster D, Prusiner SB. Chimeric prion protein expression in cultured cells and transgenic mice. Protein Sci. 1992;1:986–97. DOI: 10.1002/pro.5560010804
22. Race BL, Meade-White KD, Ward A, Jewell J, Miller MW, Williams ES, et al. Levels of abnormal prion protein in deer and elk with chronic wasting disease. Emerg Infect Dis. 2007;13:824–30.
23. Kascsak RJ, Rubenstein R, Merz PA, Tonna-DeMasi M, Fersko R, Carp RI, et al. Mouse monoclonal and polyclonal antibody to scrapie-associated fibril proteins. J Virol. 1987;61:3688–93.
24. Matsunaga Y, Peretz D, Williamson A, Burton D, Mehlhorn I, Groth D, et al. Cryptic epitopes in N–terminally truncated prion protein are exposed in the full-length molecule: dependence of conformation on pH. Proteins. 2001;44:110–8. DOI: 10.1002/prot.1077
25. Vorberg I, Buschmann A, Harmeyer S, Saalmuller A, Pfaff E, Groschup MH. A novel epitope for the specific detection of exogenous prion proteins in transgenic mice and transfected murine cell lines. Virology. 1999;255:26–31. DOI: 10.1006/viro.1998.9561
26. Race B, Meade-White K, Oldstone MB, Race R, Chesebro B. Detection of prion infectivity in fat tissues of scrapie-infected mice. PLoS Pathog. 2008;4:e1000232. DOI: 10.1371/journal.ppat.1000232
27. Kercher L, Favara C, Striebel JF, Lacasse R, Chesebro B. Prion protein expression differences in microglia and astroglia influence scrapie-induced neurodegenration in the retina and brain of transgenic mice. J. Virol. 2007;81:10340–51. DOI: 10.1128/JVI.00865-07
28. Schätzl HM, Da Costa M, Taylor L, Cohen FE, Prusiner SB. Prion protein variation among primates. J Mol Biol. 1995;245:362–74. DOI: 10.1006/jmbi.1994.0030
29. O'Rourke KI, Spraker TR, Hamburg LK, Besser TE, Brayton KA, Knowles DP. Polymorphisms in prion precursor functional gene but not the pseudogene are associated with susceptibility to chronic wasting disease in white-tailed deer. J Gen Virol. 2004;85:1339–46. DOI: 10.1099/vir.0.79785-0
30. Fox KA, Jewell JE, Williams ES, Miller MW. Patterns of PrPCWD accumulation during the course of chronic wasting disease infection in orally inoculated mule deer (*Odocoileus hemionus*). J Gen Virol. 2006;87:3451–61. DOI: 10.1099/vir.0.81999-0
31. Schneider I, Schneider H, Schneider MP, Silva A. The prion protein and New World primate phylogeny. Genet Mol Biol. 2004;27:505–10. DOI: 10.1590/S1415-47572004000400007
32. Gibbs CJ Jr, Gajdusek DC. Experimental subacute spongiform virus encephalopathies in primates and other laboratory animals. Science. 1973;182:67–8. DOI: 10.1126/science.182.4107.67
33. Spraker TR, Zink RR, Cummings BA, Wild MA, Miller MW, O'Rourke KI. Comparison of histological lesions and immunohistochemical staining of proteinease-resistant prion protein in a naturally occurring spongiform encephalopathy of free-ranging mule deer (*Odocoileus hemionus*) with those of chronic wasting disease of captive mule deer. Vet Pathol. 2002;39:110–9. DOI: 10.1354/vp.39-1-110

34. Herzog C, Riviere J, Lescoutra-Etchegaray N, Charbonnier A, Leblanc V, Sales N, et al. PrPTSE distribution in a primate model of variant, sporadic, and iatrogenic Creutzfeldt-Jakob disease. J Virol. 2005;79:14339–45. DOI: 10.1128/JVI.79.22.14339-14345.2005
35. Lasmezas CI, Comoy E, Hawkins S, Herzog C, Mouthon F, Konold T, et al. Risk of oral infection with bovine spongiform encephalopathy agent in primates. Lancet. 2005;365:781–3.
36. Belay ED, Maddox RA, Williams ES, Miller MW, Gambetti P, Schonberger LB. Chronic wasting disease and potential transmission to humans. Emerg Infect Dis. 2004;10:977–84.
37. Mawhinney S, Pape WJ, Forster JE, Anderson CA, Bosque P, Miller MW. Human prion disease and relative risk associated with chronic wasting disease. Emerg Infect Dis. 2006;12:1527–35.
38. Anderson CA, Bosque P, Filley CM, Arciniegas DB, Kleinschmidt-Demasters BK, Pape WJ, et al. Colorado surveillance program for chronic wasting disease transmission to humans: lessons from 2 highly suspicious but negative cases. Arch Neurol. 2007;64:439–41. DOI: 10.1001/archneur.64.3.439

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Distant Relatives of Severe Acute Respiratory Syndrome Coronavirus and Close Relatives of Human Coronavirus 229E in Bats, Ghana

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We tested 12 bat species in Ghana for coronavirus (CoV) RNA. The virus prevalence in insectivorous bats (n = 123) was 9.76%. CoV was not detected in 212 fecal samples from *Eidolon helvum* fruit bats. Leaf-nosed bats pertaining to *Hipposideros ruber* by morphology had group 1 and group 2 CoVs. Virus concentrations were ≤45,000 copies/100 mg of bat feces. The diversified group 1 CoV shared a common ancestor with the human common cold virus hCoV-229E but not with hCoV-NL63, disputing hypotheses of common human descent. The most recent common ancestor of hCoV-229E and GhanaBt-CoVGrp1 existed in ≈1686–1800 AD. The GhanaBt-CoVGrp2 shared an old ancestor (≈2,400 years) with the severe acute respiratory syndrome–like group of CoV.

Coronaviruses (CoVs) (order Nidovirales, family *Coronaviridae*, genus *Coronavirus*) are enveloped viruses with plus-stranded RNA genomes of 26–32 kb, the largest contiguous RNA genomes in nature (*1*). They are classified into 3 groups, which contain viruses pathogenic for mammals (groups 1 and 2) and poultry (group 3) (*1*). Human CoVs (hCoVs)-229E, -NL63, -OC43, and -HKU1 are endemic worldwide and cause mainly respiratory infections in children and adults. The severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) is a novel zoonotic coronavirus that caused an international epidemic in 2002–2003. Fortunately, efficient public health management interrupted this epidemic (*2*). Studies conducted in China in the aftermath of the SARS epidemic have identified CoVs in bats (Chiroptera) and implicated this speciose mammalian order as the most likely reservoir of all known coronaviruses (*3*–*7*). Among the most urgent concerns prompted by the SARS epidemic is the likelihood of similar future events. Thus, it seems highly relevant to study the ecology of bat CoVs in terms of diversity, host restriction, virus prevalence, risk of exposure, and the circumstances of past host transition events.

The genetic diversity of bat-borne CoVs is currently unclear. Preliminary data suggest that CoVs may be adapted in a stricter sense to a specific host species rather than to specific regions (*5,6,8*–*12*). A variety of pathogenic CoVs occur in other mammals or poultry. However, the genetic range within these animals is considerably less than that observed in even single bat species or subfamilies (*7,8*).

Estimates indicate that there are ≥100 bat species in sub-Sahran Africa. This finding is in contrast to ≈50 species in the entire Western Palaearctic region (Europe, Middle East, North Africa) (*13,14*). African bats have been shown to harbor pathogens that are occasionally transmitted to humans. This transmission may result in severe disease outbreaks, e.g., Ebola and Marburg viruses (*15*). Because bats are a part of the human diet in wide areas of Africa (*16*), it appears highly relevant to study CoVs in African bats.

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DOI: 10.3201/eid1509.090224

We have demonstrated by serologic studies that African bats have antibodies against CoVs (*10*). Antibodies reactive with SARS-CoV antigen were detected in 47 (6.7%) of 705 bat serum specimens from 26 species (*10*). Recently, Tong et al. detected sequences of CoVs in bats from Kenya (*17*). We describe the results of studies on bats in Ghana obtained by using noninvasive sampling of frugivorous and insectivorous bats at 2 caves, a lake habitat of diverse insectivorous bats, and a large urban roosting site of frugivorous bats. Bayesian inference of diversification dates gave implications on the recency of the introduction of hCoV-229E into the human population, irrespective of its original source.

Materials and Methods

Capturing and Sampling

In the locations identified in Figure 1, mist netting and sampling were conducted as described (*11*). In Kumasi Zoo, fecal samples were collected with plastic foil under trees occupied by *Eidolon helvum* bats (estimated colony size 300,000). For all capturing and sampling, permission was obtained from the Wildlife Division of the Ministry of Lands, Forestry, and Mines in Ghana. Research samples were exported under a state agreement between the Republic of Ghana and the Federal Republic of Germany, represented by the City of Hamburg. Additional export permission was obtained from the Veterinary Services of the Ghana Ministry of Food and Agriculture.

Processing and Analysis of Samples

Samples (1–4 fecal pellets or swabs suspended in RNA stabilization solution [RNAlater Tissue Collection; Applied Biosystems, Foster City, CA, USA]) were tested at the Kumasi Centre for Collaborative Research in Tropical Medicine as described (*11,18*). After initial sequencing, specific primers were designed for each group of CoV found. Nested reverse transcription–PCR (RT-PCR) primer sets used for sequencing of longer fragments of representative viruses are available upon request. The following sequences were derived from this study and were submitted to GenBank under the listed accession numbers: RNA-dependent RNA polymerase (RdRp) sequences: BtCoV/Hip/GhanaBoo/348/2008, FJ710043; BtCoV/Hip/GhanaBoo/344/2008, FJ710044; BtCoV/Hip/GhanaKwam/8/2008, FJ710045; BtCoV/Hip/GhanaKwam/19/2008, FJ710046; BtCoV/Hip/GhanaKwam/20/2008, FJ710047; BtCoV/Hip/GhanaKwam/13/2008, FJ710048; BtCoV/Hip/GhanaKwam/31/2008, FJ710049; BtCoV/Hip/GhanaKwam/27/2008, FJ710050; BtCoV/Hip/GhanaKwam/26/2008, FJ710051; BtCoV/Hip/GhanaKwam/24/2008, FJ710052; BtCoV/Hip/GhanaKwam/10/2008, FJ710053; BtCoV/Hip/GhanaKwam/22/2008, FJ710054/nucleocapsid sequences; BtCoV/Hip.sp/GhanaBoo/344/2008, FJ710055; BtCoV/Hip.sp/GhanaKwam/19/2008, FJ710056.

Phylogenetic Analysis

Nucleic acid alignments were conducted based on amino acid code by using the ClustalW algorithm (www.ebi.ac.uk/clustalw) in the Molecular Evolutionary Genetics Analysis version 4.0 software package (www.megasoftware.net) (*19*). Two gap-free nucleotide alignments (817 bp and 1,221 bp) were generated. Tree topologies were determined on both datasets by using MrBayes version 3.1 (*20*). The analysis used a general time reversible (GTR) substitution model, with 6 rate categories to approximate a gamma-shaped rate distribution across sites and an invariant site assumption (GTR + Γ6 + I). Markov chain Monte Carlo (MCMC) chains of 10^7 iterations were sampled every 500 generations, resulting in 20,000 sampled trees. Two

Figure 1. Location of Kwamang caves near the village of Kwamang, (6°58′N, 1°16″W), 50 km northeast of Kumasi, Ashanti region, Ghana. Booyem caves A (7°43′24.9″N, 1°59′16.5″W) and B (7°43′25.7″N, 1°59′33.5″W) are located near remote small settlements in the vicinity of Booyem, Brong-Ahafo region. Lake Bosumtwi is located 30 km southeast of Kumasi (6°32′22.3″N, 1°24′41.5″W). The botanical gardens of Kwame Nkrumah National University of Science and Technology are located on campus in the city of Kumasi (6°41′6.4″N, 1°33′42.8″W). Kumasi Zoo is located in the center of the city (6°42′2.0″N, 1°37′29.9″W).

Metropolis-coupled chains (1 cold and 3 heated chains each) were run in parallel, compared, and pooled. Convergence of chains was confirmed by the potential scale reduction factor statistic in MrBayes (*21*) and by visual inspection of each cold chain using the TRACER program (*22*). Phylogenetic dating was conducted by using Bayesian evolutionary analysis sampling trees (BEAST) (*22*). Chain lengths in BEAST were at least 20,000,000 generations with sampling every 500 generations. Convergence of the model was checked visually and by the effective sample size statistic with TRACER.

Results

Virus Detection

During February 2008, bats were sampled in the described locations around Kumasi, Ghana. Initially, 7 fecal samples tested positive by pan-CoV PCR. Products (440 bp, *RdRp* gene) were sequenced and aligned with prototype CoV. Neighbor-joining phylogenies indicated 2 distinct groups of sequences that belonged to CoV group 1 (n = 4) and group 2 (n = 3), respectively. Specific primer pairs for the group 1 and group 2 sequences were designed and applied again to all samples. Five additional viruses were found, resulting in a total CoV prevalence of 9.76% in insect-eating bats (n = 123). No virus was found in any oral swab. All virus findings in fecal samples are listed by capture site in Table 1.

Notably, all CoV findings were in insect-eating leaf-nosed bats of the genus *Hipposideros*. Within the genus, the species *H. abae* could be discriminated unambiguously by morphology (Table 1). The remaining *Hipposideros* species were assigned to the complex of forms related to currently recognized species *H. caffer* and *H. ruber*. Because 2 morphotypes were present (Figure 2), the mitochondrial cytochrome *b* gene was sequenced as described (*23*). Both morphotypes belonged to phylogenetic lineages distinct from *H. caffer* and possibly represented 2 distinct species (P. Vallo, personal ongoing investigation). Both are collectively referred to as *H. caffer* (*cf.*) *ruber* in this study. A fraction of 15.4 % of *H. cf. ruber* specimens yielded CoV, without a difference between sexes (14%/19%, n = 57/21 [M/F], respectively). Only adult males and nonlactating adult females, but no lactating females, juveniles, and subadults of *H. cf. ruber* were encountered.

Virus Concentrations

To estimate the quantity of CoV genomes in bat feces, we did end-point dilution experiments with the nested pan-CoV RT-PCR (*18*). The previously determined sensitivity limit of the PCR assay was 5–45 copies/PCR (*18*). In the assay, the equivalent of 1 mg feces was tested per PCR tube (100 mg feces collected, 1:10 dilution extracted, 1:10 dilution tested). The highest dilution factor that still yielded an amplification signal in any of the samples was 1:10, which suggested a maximal concentration of 50 to 450 CoV RNA copies/mg of feces.

Virus Classification

Group 1 CoV

In *H. cf. ruber* bats in the Kwamang and Booyem caves, a diverse group 1 CoV was found. Further analysis was complicated by the low RNA content in samples. Based on alignments of prototype group 1 viruses, 5 different nested RT-PCRs were designed and the *RdRp* fragment could finally be extended by 441 bp to the 5′ end, providing

Table 1. Overview of bats studied, Ghana

Sampling site	Species	No. fecal samples	No. positive (group 1/group 2)
Zoo Kumasi (6°42′2.0″N, 1°37′29.9″W)	*Eidolon helvum*	212*	0
KNUST Botanical Garden Kumasi (6°41′6.4″N, 1°33′42.8″W)†	*Pipistrellus nanulus*	1	0
	Glauconycteris beatrix	1	0
Lake Bosumtwi (6°32′22.3″N, 1°24′41.5″W)	*Chaerephon spp.*	6	0
	Nycteris hispida	1	0
	P. nanus	5	0
	P. deserti	1	0
Cave Kwamang (6°58′N, 1°16′W)	*Hipposideros caffer ruber*	40	10 (4/6)
	H. abae	13	0
Cave Booyem A (7°43′24.9″N, 1°59′16.5″W)	*H. cf. ruber*	8	0
	Coleura afra	12	0
Cave Booyem B (7°43′25.7″N, 1°59′33.5″W)	*H. cf. ruber*‡	11	2 (1/1)
	H. abae	3	0
	Coleura afra	21	0
Total		335	12

*These samples were collected without individual association to bats. Due to a low sampling frequency, it can be assumed that each sample was from an individual bat.
†Kwame Nkrumah University of Science and Technology.
‡Two morphotypes were observed.

Figure 2. Two morphotypes of *Hipposideros caffer ruber*, held by one of the authors (F.G.-R.), who was wearing a leather glove. Photograph courtesy of Antje Seebens.

an 817-nt fragment for phylogenetic analysis. All methods of phylogenetic inference placed this virus next to a common ancestor with human coronavirus 229E, which circulates worldwide in humans (Figure 3). Bootstrap support of the hCoV-229E/GhanaBt-CoVGrpI root point in neighbor-joining analysis was 100%. The corresponding Bayesian posterior probability was 1.0. The most closely related member of the GhanaBt-CoVGrp1 clade shared 91.90% nucleotide identity with hCoV-229E in the analyzed fragment. The most distant member was 86.50% identical. The next phylogenetic neighbor, the human CoV hCoV-NL63, was only 74.70%–78.60% identical in the analyzed fragment.

Group 2 CoV

With the pan-CoV screening assay, a group 2 CoV was initially found in the Kwamang cave. Sequences from 3 bats were identical. The secondary group-specific PCR identified 4 additional samples of this virus, 1 of them from Booyem Cave B and the remaining from Kwamang. Nucleotide identity among these sequences was 97.2%–100%. Phylogenetic analysis with different methods of inference (neighbor-joining nucleotide-based, neighbor-joining amino acid–based, Bayesian) yielded variable tree topologies suggesting basal associations with either the 2a, 2d, or 2b subgroups (data not shown) (*24*). Based on alignments of prototype group II viruses, 8 additional nested RT-PCR primer sets were designed and 2 of the samples could be

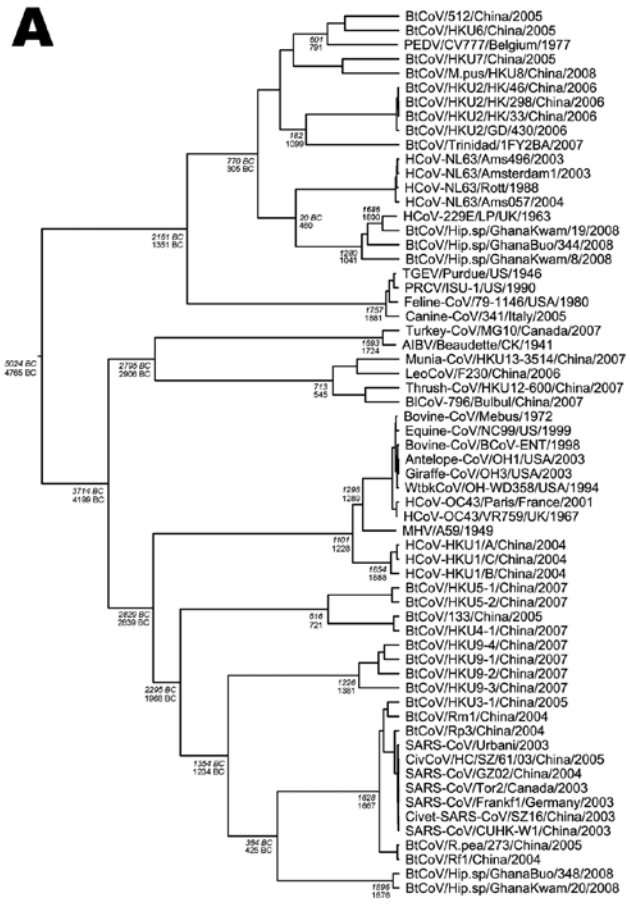

Figure 3. A) Phylogeny of coronaviruses (CoVs) in the RNA-dependent RNA polymerase gene (RdRp, 817-bp fragment) with root point dates derived from Bayesian inference under a relaxed lognormal molecular clock assumption with a codon-based substitution model (SRD06) and an assumption of expansion growth of the virus population. Estimated dates of diversification of CoV lineages at root points are shown in italics for the expansion growth population model and in regular type for the exponential growth model. Dates BC are identified with a suffix; dates AD are not. B) Bayesian phylogeny from the CoV group 1 root, using the nucleocapsid (N) gene. Highest posterior densities for all root points were >0.99, except where indicated.

amplified. Sequences could be extended 520 bp upstream and 383 bp downstream of the initial fragments, yielding 1,221-bp fragments for phylogenetic analysis. Bayesian phylogenetic inference with different substitution models and parallel analysis using Metropolis coupling now placed the virus reliably next to a common ancestor with the 2b group of CoV (SARS-like viruses, Figure 3). The Bayesian posterior probability of the CoV 2b/GhanaBt-CoVGrp2 clade being monoyphletic was 1.0. A maximum of 72.2% nucleotide identity was shared with SARS CoV.

Molecular Dating

Reliable isolation dates were researched in the literature for each employed virus. Because a reliable molecular clock dating existed for the most recent common ancestor (MRCA) of the hCoV-OC43/bovine CoV pair (*25*), this date was set as a normal-distributed probabilistic prior within the published ranges (*25*) for calibration of all analyses. A first analysis was conducted on the 1,225-bp dataset that did not include the novel GhanaBt-CoVGrp1. All virus sequences were assumed to be contemporary. Phylogeny was inferred using a GTR + Γ4 + I model. The resulting MRCA date of the CoV2b (SARS-like)/GhanaBt-CoVGrp2 clade was 260 AD and that of the hCoV-NL63/-229E pair was 981 AD (see Table 2 for details). To include the novel GhanaBat-CoVGrp1, we repeated the same analysis by using the 817-bp dataset. The resulting MRCA date of the hCoV-NL63/229E pair was 816 AD in this analysis, which was in good concordance with results from the 1,221-bp dataset (Table 2) and also with previously published data (*26*). The diversification estimate for the novel group 1 bat-CoV and hCoV-229E then was 1803 AD.

Because it has been suggested that codon-based evolutionary models may be preferred for Bayesian phylogenetic inference from protein-coding datasets (*27*), analyses on the 817-bp dataset were repeated by using the SRD06 substitution model in BEAST. This analysis did not yield a different substitution rate, but resulted in older resulting MRCA dates (Table 2). A Bayes factor test conducted in TRACER yielded a strong estimate of superiority of the codon-based model over the GTR + Γ4 + I model ($\log_{10}$ Bayes factor 139 [20 is highly significant]). To further optimize the prediction of MRCA dates, the constant population size assumption used in all analyses was exchanged against expansion growth or exponential growth assumptions. Both assumptions were predicted to fit the data better than the constant size model (Bayes factors 13.5 and 13.9). There was no difference between the expansion and exponential models (Bayes factor 0.34 in favor of expansion). The MRCA date of hCoV-229E and the GhanaBt-CoVGrp1 was 1686 (expansion) or 1800 (exponential growth). Table 2 summarizes the results. Figure 3 shows a dated phylogeny of coronaviruses with MRCAs according to the 2 last mentioned analyses.

Recombination

To determine whether CoV recombination might play a role in the studied virus population, the structural nucleocapsid gene was amplified using 8 nested RT-PCR primer sets that had been designed on alignments of all available CoV group 1 nucleocapsid sequences. Using a similar approach, we also tested the same samples for CoV group 2 nucleocapsid sequences. Only group 1 RT-PCRs yielded fragments. These fragments could be combined into contig-

Table 2. Results of molecular clock analyses, study of coronaviruses in bats, Ghana*

Alignment, bp	Mean substitution rate (substitutions/ site/year)	Population model, substitution model	Root point (range)†	MRCA (95% CI, HPD)‡			
				SARS-like§	SARS-like/ GhanaBt-CoVGrp2	hCoV-229E/ hCoV-NL63	HCoV-229E/ GhanaBt-CoVGrp1
1,221	2.1×10^{-4} ($1.2–3.1 \times 10^{-4}$)	Constant size, nucleotide (GTR + G + I)	2243 (4521–290)	1905 AD (1867–1941)	260 (792 BC–1178)	981 (161 BC–1324)	–
817	2.1×10^{-4} ($1.5–2.7 \times 10^{-4}$)	Constant size, nucleotide (GTR + G + I)	2053 (3433–731)	1852 AD (1612–1852)	348 (119 BC–820)	816 (320–1290)	1803 (1694–1906)
817	1.6×10^{-4} ($0.8–2.5 \times 10^{-4}$)	Constant size, codon-based (SRD06)	4500 (7305–1918)	1674 (1516–1804)	768 BC (2037 BC–360)	168 (1111 BC–721)	1659 (1503–1804)
817	1.5×10^{-4} ($0.9–2.2 \times 10^{-4}$)	Expansion growth, codon-based (SRD06)	5024 (9261–1360)	1628 (1379–1836)	384 BC (2060 BC–1074)	20 (1347 BC–1174)	1686 (1460–1871)
817	1.8×10^{-4} ($0.9–2.8 \times 10^{-4}$)	Exponential growth, codon-based (SRD06)	4765 (7999–1707)	1667 (1436–1853)	425 (1544 BC–1193)	460 (956 BC–1271)	1800 (1501–1883)

*MRCA, most recent common ancestor; CI, confidence interval; HPD, high population density; SARS, severe acute respiratory syndrome; hCoV, human coronavirus; GTR + Γ + I, general time reversible gamma-shaped rate distribution across sites and an invariant site assumption.
†Estimation of the year (BC) of the most recent common ancestor.
‡Estimation of the year of the most recent common ancestor of extant CoV. All years AD except as indicated.
§CoV group 2b without novel Bt-CoV from this study (Figure 2).

uous 1,030-nt sequences for Bt-CoV GhanaKwam 19 and 1,176 nt for Bt-CoV GhanaBoo 344. As shown in Figure 3, panel B, the resulting phylogenetic placement was exactly matching that of the *RdRp* fragments, giving no evidence of recombination between the *RdRp* region located in the middle of the genome and the nucleocapsid gene located at the extreme downstream end. Sequencing of the nucleocapsid gene of the GhanaBt-CoVGrp2 was not successful when we used 15 nested RT-PCRs designed on alignments of all available CoV 2b nucleocapsid sequences. Amplification with above mentioned nested RT-PCRs for CoV group 1 was also unsuccessful.

Discussion

In the aftermath of the SARS epidemic, bats have been identified as carriers of CoV in China (*3*–*7*). Furthermore, in addition to our earlier finding of antibodies against CoVs in various African bats (*10*), we have confirmed the presence of CoV in bats of Ghana. Together with recent data from Germany, North America, Trinidad, and Kenya (*11,12,28*), these findings suggest that the association of CoV with bats is a worldwide phenomenon. The prevalence of CoV in insect-eating bats (9.76%) matched our previous findings in Germany. However, in that study we sampled during the breeding season and showed that CoVs are most likely amplified in maternity roosts (*11*). The composition of the catch in this study (no lactating females, no young bats) suggests sampling outside the breeding season and may not be directly comparable. Future studies relating to risks of exposure should address whether virus prevalence may change over time.

The risk of exposure was also addressed by investigations of virus concentration. Several groups have shown that CoVs are almost exclusively detected in bat feces and not, as hypothesized earlier, in saliva (*3,4,28,29*). Surprisingly, little virus was found in all fecal samples tested in our study. We estimated the RNA concentration per full sample (100 mg feces = 2–4 fecal pellets) to be only up to 4.5×10^4 RNA copies. Human pathogenic viruses transmitted by the fecal-oral route generate much higher virus concentrations in stool, up to $\approx 10^{12}$ RNA copies/mg, e.g., for different picornaviruses (*30*–*32*). Based on these data it would be difficult to postulate that humans can acquire CoV from bat feces. However, studies in other locations and at different times are needed to address virus concentration in bat droppings in more detail. Because virus in this study was only observed in insectivorous bats and not in frugivorous bats, future studies should investigate whether insects might constitute a source of CoV infection for bats.

To achieve a direct prediction of the potential of bat CoVs to infect human cells, it would be highly relevant to conduct virus isolation studies on bat feces. However, in our study we sampled no more than 100 mg of feces per bat. All samples had to be collected in RNAlater solution (0.5 mL) (Applied Biosystems) for reasons of storage and transportation. Although it has been suggested that RNAlater solution may preserve virus infectivity (*33,34*), our observations showed that the solution has to be diluted at least 1:20 in cell culture medium to avoid cytotoxicity (data not shown). Because of the low virus RNA concentrations observed, we did not attempt to isolate the virus. However, the absence of successful virus isolation from bat feces in previous studies (*3–6,8,11,12*) may not reflect the incapability of bat CoV to infect human cells. Recently, a synthetic bat CoV complemented with an appropriate spike protein has shown potential to infect human cells (*35*).

Reconstruction of phylogenetic and temporal relationships between bat CoV and other mammalian CoV is another way to obtain information on their zoonotic potential. Unfortunately, for CoV long sequence fragments must be analyzed before valid phylogenies can be inferred from the conserved nonstructural genome portion (*28,36*). Because of the low concentration of RNA in bat samples, generation of long sequences from novel bat CoV is tedious and technically demanding, which may be why some published phylogenies of bat CoV are based on short datasets, making it difficult to use these data for reference. For molecular clock dating, we have therefore relied on reference viruses mainly from other mammals that covered our 1,221-bp fragment in the conserved *RdRp* region. We assumed that the *RdRp* would be under less selective pressure than the structural genes and other nonstructural genes, and therefore could be used to infer nucleotide substitution rates over distantly related CoVs (*7,25,26,36–38*). We have confirmed all tree topologies using alternative methods of phylogenetic inference, including an MCMC algorithm implemented in MrBayes that eliminates artifacts contributed by fixation of MCMC chains in suboptimal prosterior probability maxima (*20*). Calibration was conducted on reliable isolation dates of prototype and novel bat CoV from the literature, as well as on the MRCA of the hCoV-OC43/Bovine CoV clade. For dating of only this specific CoV clade, a wide range of dated virus isolates has been available that covered as much as 34% (1965–2004) of the projected time of virus evolution from root to tip (1890–2004) (*25*). A probabilistic calibration prior was used, which is favorable for dating in combination with relaxed molecular clock assumptions (*39*). The determined mean substitution rates were in good concordance with earlier studies on non-bat–CoV that used maximum likelihood-based methods in addition to Bayesian inference (*25,26,38,40*).

Although the exponential growth prior on the virus population seemed equivalent with an expansion growth model by the Bayes factor test and produced highly compatible MRCAs, the exponential model produced a better match with the previously determined MRCA of the

HCoV-NL63/HCoV-229E pair (*26*). Because Pyrc et al. generated these data by 3 alternative approaches (Bayesian, serial unweighted pair group method with arithmetic mean, maximum likelihood [*26*]), we used their MRCA to validate our results, and consequently prefer the MRCA dating from the exponential growth population model (as presented in Figure 3 in plain type). One earlier study on bat and non-bat CoV suggested a much faster evolutionary rate for CoV than other studies (*7*). As Vijaykrishna et al. pointed out, their results were associated with large confidence intervals caused by the lack of available data on Bt CoV at the time the study was conducted (*7*). The increase of available sequence data now enables a better account of CoV evolutionary history.

All CoVs in our study were found in members of the genus *Hipposideros* (family Hipposideridae). The genus *Rhinolophus* from the sister family Rhinolophidae was found to host SARS-like viruses in several studies in China. One of our *Hipposideros* CoVs was in a basal phylogenetic relationship with the SARS-like clade (group 2b); their most recent common ancestors date back to ≈400 BC. Tong et al. (*17*) have detected a sequence fragment of a bat CoV in Kenya that also belongs to the 2b clade but is associated with the genus *Chaerephon*, a free-tailed bat that is rather distantly related to the genus *Hipposideros*. Although these authors analyzed only a short sequence fragment, their 2b CoV seems to be related more closely to SARS CoV than the virus found in our study. In the many studies conducted in China, only closely related members of the 2b group were detected, with the most basal members dating back only to the 17th century, according to our analysis. The cooccurrence of basal and closely related viruses in Africa, as well as the existence of the same virus clade in bats other than those of the family Hipposideridae, may entail speculations about a possible origin of the SARS-like group of CoVs in Africa rather than in Asia.

Another result that should be integrated with earlier findings is the surprisingly recent date of the MRCA of the novel Grp1 Bt CoV and the human common cold virus hCoV-229E. Further to the proven recent host switching of SARS CoV, Vijgen et al. have suggested that hCoV-OC43 entered the human population ≈120 years ago, causing a pandemic (*25*). This virus was most likely acquired by humans from domestic cattle. Results of our study show that it is not unlikely that hCoV-229E, which today is circulating worldwide in humans, resulted from a host switching event not more than 208–322 years ago. However, as with molecular clock dating of viruses, associated confidence limits should not be overlooked.

Because *H. cf. ruber* bats are found only in sub-Saharan Africa and are not migratory (*23*), it would be relevant to know how tightly the associated CoV is restricted to its host. Despite the statistical limitations of our rather small sample size, the absence of CoV in bats of the closely related species *H. abae* that were tested in our study in 2 different caves speaks in favor of tight host restriction. Another supportive argument is the absence of CoV in *C. afra*, a bat species sampled in sufficient numbers at the Booyem cave. This cave was coinhabited by CoV-positive *H. cf. ruber* bats. If tight host restriction to nonmigratory *H. cf. ruber* bats existed, this would indicate an origin of hCoV-229E within the geographic range of its host, i.e., the rainforest belt and the wet forested savannahs of sub-Saharan Africa (*23*). Unfortunately, it will be difficult to reconstruct whether the projected host transition event might have been associated with human epidemic disease.

Acknowledgment

We are grateful to the voluntary staff at Noctalis for support.

This study was supported by the German Ministry of Education and Research (Project Code Ökologie und Pathogenese von SARS), the European Commission (contracts SSPE-CT-2005-022639 and FP7-INFRASTRUCTURES-2008-No 228292), and the Bundesamt für Bevölkerungsschutz und Katastrophenhilfe (contract BBK-F-440-00-1).

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References

1. Cavanagh D. Nidovirales: a new order comprising *Coronaviridae* and *Arteriviridae*. Arch Virol. 1997;142:629–33.
2. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003;348:1967–76. DOI: 10.1056/NEJMoa030747
3. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc Natl Acad Sci U S A. 2005;102:14040–5. DOI: 10.1073/pnas.0506735102
4. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. Science. 2005;310:676–9. DOI: 10.1126/science.1118391
5. Poon LL, Chu DK, Chan KH, Wong OK, Ellis TM, Leung YH, et al. Identification of a novel coronavirus in bats. J Virol. 2005;79:2001–9. DOI: 10.1128/JVI.79.4.2001-2009.2005
6. Tang XC, Zhang JX, Zhang SY, Wang P, Fan XH, Li LF, et al. Prevalence and genetic diversity of coronaviruses in bats from China. J Virol. 2006;80:7481–90. DOI: 10.1128/JVI.00697-06
7. Vijaykrishna D, Smith GJ, Zhang JX, Peiris JS, Chen H, Guan Y. Evolutionary insights into the ecology of coronaviruses. J Virol. 2007;81:4012–20. DOI: 10.1128/JVI.02605-06
8. Woo PC, Lau SK, Li KS, Poon RW, Wong BH, Tsoi HW, et al. Molecular diversity of coronaviruses in bats. Virology. 2006;351:180–7. DOI: 10.1016/j.virol.2006.02.041
9. Wong S, Lau S, Woo P, Yuen KY. Bats as a continuing source of emerging infections in humans. Rev Med Virol. 2007;17:67–91. DOI: 10.1002/rmv.520

10. Muller MA, Paweska JT, Leman PA, Drosten C, Grywna K, Kemp A, et al. Coronavirus antibodies in African bat species. Emerg Infect Dis. 2007;13:1367–70.
11. Gloza-Rausch F, Ipsen A, Seebens A, Gottsche M, Panning M, Felix Drexler J, et al. Detection and prevalence patterns of group I coronaviruses in bats, northern Germany. Emerg Infect Dis. 2008;14:626–31. DOI: 10.3201/eid1404.071439
12. Dominguez SR, O'Shea TJ, Oko LM, Holmes KV. Detection of group 1 coronaviruses in bats in North America. Emerg Infect Dis. 2007;13:1295–300.
13. Proches S. The world's biogeographical regions: cluster analyses based on bat distributions. J Biogeogr. 2005;32:607–14. DOI: 10.1111/j.1365-2699.2004.01186.x
14. Mayer F, Dietz C, Kiefer A. Molecular species identification boosts bat diversity. Front Zool. 2007;4:4. DOI: 10.1186/1742-9994-4-4
15. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T. Bats: important reservoir hosts of emerging viruses. Clin Microbiol Rev. 2006;19:531–45. DOI: 10.1128/CMR.00017-06
16. Swensson J. Bushmeat trade in Techiman, Ghana, West Africa. Uppsala (Sweden): Uppsala University; 2005.
17. Tong S, Conrardy C, Ruone S, Kuzmin IV, Guo X, Tao Y, et al. Detection of novel SARS-like and other coronaviruses in bats from Kenya. Emerg Infect Dis. 2009;15:482–5. DOI: 10.3201/eid1503.081013
18. de Souza Luna LK, Heiser V, Regamey N, Panning M, Drexler JF, Mulangu S, et al. Generic detection of coronaviruses and differentiation at the prototype strain level by reverse transcription-PCR and nonfluorescent low-density microarray. J Clin Microbiol. 2007;45:1049–52. DOI: 10.1128/JCM.02426-06
19. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
20. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572–4. DOI: 10.1093/bioinformatics/btg180
21. Gelman A, Rubin DB. Markov chain Monte Carlo methods in biostatistics. Stat Methods Med Res. 1996;5:339–55. DOI: 10.1177/096228029600500402
22. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007;7:214. DOI: 10.1186/1471-2148-7-214
23. Vallo P, Guillén-Servent A, Benda P, Pires DB, Koubek P Variation of mitochondrial DNA reveals high cryptic diversity in *Hipposideros caffer* complex. Acta Chiropt. 2008;10:193–206. DOI: 10.3161/150811008X414782
24. Woo PC, Wang M, Lau SK, Xu H, Poon RW, Guo R, et al. Comparative analysis of twelve genomes of three novel group 2c and group 2d coronaviruses reveals unique group and subgroup features. J Virol. 2007;81:1574–85. DOI: 10.1128/JVI.02182-06
25. Vijgen L, Keyaerts E, Moes E, Thoelen I, Wollants E, Lemey P, et al. Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. J Virol. 2005;79:1595–604. DOI: 10.1128/JVI.79.3.1595-1604.2005
26. Pyrc K, Dijkman R, Deng L, Jebbink MF, Ross HA, Berkhout B, et al. Mosaic structure of human coronavirus NL63, one thousand years of evolution. J Mol Biol. 2006;364:964–73. DOI: 10.1016/j.jmb.2006.09.074
27. Shapiro B, Rambaut A, Drummond AJ. Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. Mol Biol Evol. 2006;23:7–9. DOI: 10.1093/molbev/msj021
28. Carrington CV, Foster JE, Zhu HC, Zhang JX, Smith GJ, Thompson N, et al. Detection and phylogenetic analysis of group 1 coronaviruses in South American bats. Emerg Infect Dis. 2008;14:1890–3. DOI: 10.3201/eid1412.080642
29. Dobson AP. Virology. What links bats to emerging infectious diseases? Science. 2005;310:628–9. DOI: 10.1126/science.1120872
30. Baumgarte S, de Souza Luna LK, Grywna K, Panning M, Drexler JF, Karsten C, et al. Prevalence, types, and RNA concentrations of human parechoviruses, including a sixth parechovirus type, in stool samples from patients with acute enteritis. J Clin Microbiol. 2008;46:242–8. DOI: 10.1128/JCM.01468-07
31. Drexler JF, Luna LK, Stocker A, Almeida PS, Ribeiro TC, Petersen N, et al. Circulation of 3 lineages of a novel Saffold cardiovirus in humans. Emerg Infect Dis. 2008;14:1398–405. DOI: 10.3201/eid1409.080570
32. Takanashi S, Hashira S, Matsunaga T, Yoshida A, Shiota T, Tung PG, et al. Detection, genetic characterization, and quantification of norovirus RNA from sera of children with gastroenteritis. J Clin Virol. 2009;44:161–3. DOI: 10.1016/j.jcv.2008.11.011
33. Forster JL, Harkin VB, Graham DA, McCullough SJ. The effect of sample type, temperature, and RNAlater on the stability of avian influenza virus RNA. J Virol Methods. 2008;149:190–4.
34. Uhlenhaut C, Kracht M. Viral infectivity is maintained by an RNA protection buffer. J Virol Methods. 2005;128:189–91. DOI: 10.1016/j.jviromet.2005.05.002
35. Becker MM, Graham RL, Donaldson EF, Rockx B, Sims AC, Sheahan T, et al. Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. Proc Natl Acad Sci U S A. 2008;105:19944–9. DOI: 10.1073/pnas.0808116105
36. Snijder EJ, Bredenbeek PJ, Dobbe JC, Thiel V, Ziebuhr J, Poon LL, et al. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J Mol Biol. 2003;331:991–1004. DOI: 10.1016/S0022-2836(03)00865-9
37. Bredenbeek PJ, Snijder EJ, Noten FH, den Boon JA, Schaaper WM, Horzinek MC, et al. The polymerase gene of corona- and toroviruses: evidence for an evolutionary relationship. Adv Exp Med Biol. 1990;276:307–16.
38. Salemi M, Fitch WM, Ciccozzi M, Ruiz-Alvarez MJ, Rezza G, Lewis MJ. Severe acute respiratory syndrome coronavirus sequence characteristics and evolutionary rate estimate from maximum likelihood analysis. J Virol. 2004;78:1602–3. DOI: 10.1128/JVI.78.3.1602-1603.2004
39. Drummond AJ, Ho SY, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with confidence. PLoS Biol. 2006;4:e88. DOI: 10.1371/journal.pbio.0040088
40. Sanchez CM, Gebauer F, Sune C, Mendez A, Dopazo J, Enjuanes L. Genetic evolution and tropism of transmissible gastroenteritis coronaviruses. Virology. 1992;190:92–105. DOI: 10.1016/0042-6822(92)91195-Z

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Predicting Phenotype and Emerging Strains among *Chlamydia trachomatis* Infections

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Chlamydia trachomatis is a global cause of blinding trachoma and sexually transmitted infections (STIs). We used comparative genomics of the family *Chlamydiaceae* to select conserved housekeeping genes for *C. trachomatis* multilocus sequencing, characterizing 19 reference and 68 clinical isolates from 6 continental/subcontinental regions. There were 44 sequence types (ST). Identical STs for STI isolates were recovered from different regions, whereas STs for trachoma isolates were restricted by continent. Twenty-nine of 52 alleles had nonuniform distributions of frequencies across regions ($p<0.001$). Phylogenetic analysis showed 3 disease clusters: invasive lymphogranuloma venereum strains, globally prevalent noninvasive STI strains (*ompA* genotypes D/Da, E, and F), and nonprevalent STI strains with a trachoma subcluster. Recombinant strains were observed among STI clusters. Single nucleotide polymorphisms (SNPs) were predictive of disease specificity. Multilocus and SNP typing can now be used to detect diverse and emerging *C. trachomatis* strains for epidemiologic and evolutionary studies of trachoma and STI populations worldwide.

Chlamydia trachomatis is spread by close social contact or sexual activity. Worldwide, *C. trachomatis* is the leading preventable cause of blindness and bacterial sexually transmitted infections (STIs). Various typing techniques have been developed to better understand the epidemiology and pathogenesis of chlamydial diseases. Early typing schemes used monoclonal and polyclonal antibodies directed against the major outer membrane (MOMP) (*1*), and differentiated the organism into serovars and seroclasses: B class (comprising serovars B, Ba, D, Da, E, L_2, L_2a), C class (A, C, H, I, Ia, J, Ja, K, L_1, L_3), and intermediate class (F, G). Sequencing of *ompA*, which encodes MOMP, has refined typing, detecting numerous trachoma (*2*,*3*) and sexually transmitted (*4*,*5*) serovar subtypes.

Seroclasses, however, do not correlate with disease phenotypes. For example, A, B, Ba, and C are responsible for trachoma, whereas lymphogranuloma venereum (LGV) strains, $L_{1\text{-}3}$, are associated with invasive diseases, such as suppurative lymphadenitis and hemorrhagic proctitis (*6*). Other typing techniques, such as restriction fragment length polymorphism (*7*), random amplification of polymorphic DNA, or pulsed-field gel electrophoresis (PFGE) (*8*), and amplified fragment length polymorphism (*9*) also correlate poorly with disease phenotype, and none have been standardized across laboratories.

Multilocus sequence typing (MLST) has been used to characterize strains and lineages of numerous pathogens associated with human diseases that cause serious illness and death, including *Neisseria meningitidis*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Haemophilus influenzae*. MLST uses 500–700 bp sequences of internal regions of 6–8 housekeeping genes, excluding genes suspected to be under immune selection (where there is positive selection for sequence diversity) and ribosomal RNA genes (which are multicopy and too conserved) (*10*). Advantages of MLST include its precision, allowing simple interlaboratory comparisons, good discrimination between strains, and buffering against the distorting effect of recombination on genetic relatedness. MLST data are also amenable to various population genetic analyses (*11*,*12*). Databases for >30 species are curated at www.mlst.net and pubmlst.org. In

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DOI: 10.3201/eid1509.090272

parallel with our study, 2 multilocus schemes have recently been developed for *C. trachomatis*. The first violated the above premise by including *ompA*, which is under immune selection (*13*). The second included only laboratory-adapted and 5 clinical E strains from the Netherlands (*14*).

In this work, unlike the other *C. trachomatis* MLST schemes, we used complete genomic comparisons of 7 strains from 4 species within the family *Chlamydiaceae* to identify conserved candidate housekeeping genes across the genomes. This approach ensures that the chosen loci are stable over the course of evolution, and allows for future application of a unified MLST scheme for other *Chlamydiaceae* spp. We typed a diverse worldwide collection of reference and clinical isolates from trachoma and STI populations, correlating genetic variation with geography and disease phenotype. We found disease-specific single nucleotide polymorphisms (SNPs) and a diversity of new strains including recombinant strains that occurred for *ompA* relative to housekeeping loci, following up on our recent discovery of this phenomenon at multiple loci in *Chlamydiaceae* genomes (*15*–*17*).

Methods

Reference and Clinical Samples

Nineteen *C. trachomatis* reference strains (A/SA-1, B/TW-5, Ba/Apache-2, C/TW-3, D/UW-3, Da/TW-448, E/Bour, F/IC-Cal3, G/UW-57, H/UW-4, I/UW-12, Ia/IU-4168, J/UW-36, Ja/UW-92, K/UW-31, L1/440, L2/434, L2a/TW-396, and L3/404) and 68 clinical isolates from 6 geographic locations worldwide (obtained from patients with trachoma and STIs including proctitis) were analyzed. Because de-identified clinical data and samples were used, the research was considered institutional review board exempt by Children's Hospital Oakland Research Institute.

Selection of Housekeeping Genes

We genome-sequenced 7 strains from 4 species of the 2 genera of *Chlamydiaceae*: *C. trachomatis* (strains D/UW-3/CX [*18*] and A/Har-13 [*19*]), *Chlamydia muridarum* (rodent strain MoPn [*20*]), *Chlamydophila pneumoniae* (human strains AR39 [*20*]; CWL029 [*21*], and J138 [*21*]), and *Chlamydophila caviae* (guinea pig inclusion conjunctivitis strain [*22*]), the most distantly related species of *Chlamydiaceae*. On the basis of comparative genomics (*20*) and comparisons generated by CGView (*23*), we identified an initial candidate pool of 14 housekeeping genes (Figure 1) present in all 7 genomes with an average BLAST score ratio (BSR) (*24*) >0.5 for orthologs queried against *C. caviae* relative to the BLAST score of each sequence against itself. The BSR of >0.5 provides a cutoff to select genes that have lower levels of nucleotide sequence divergence in the genome (i.e., putative housekeeping genes). We then selected 7 genes (Figure 1) on the basis of i) diverse chromosomal regions where a single recombinational exchange would be unlikely to co-introduce >1 selected gene; ii) regions where several contiguous genes were involved in metabolic or key functions; iii) essential metabolic enzymes (e.g., tRNA synthases); iv) genes without similarity to human genes; and v) no genes under diversifying selection.

ompA and MLST Analyses

DNA was extracted from isolates using Roche High Pure Kits (Roche Diagnostics, Pleasanton, CA, USA) and *ompA* genotyped as described (*15*,*16*,*25*). DNASTAR (Madison, WI, USA) was used to design primers to amplify ≈600–700 bp for each gene (Table 1); BLAST (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to ensure primer specificity for *C. trachomatis* genes. MLST PCR was carried out in 96-well plates as described (*26*). Sequenced

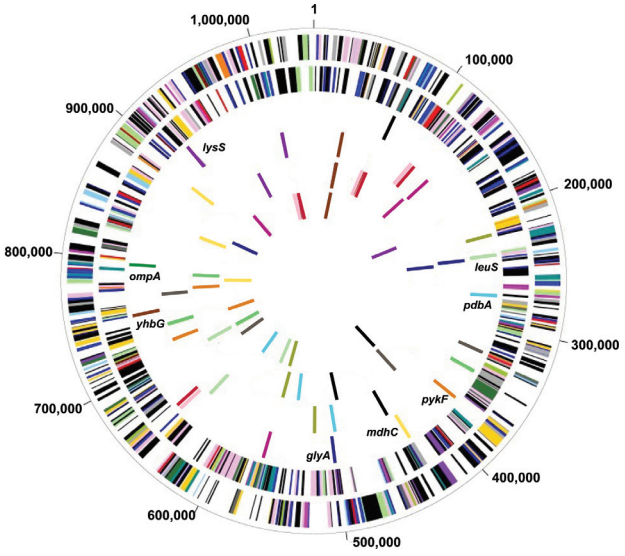

Figure 1. Comparison of 14 housekeeping genes among genome sequences of 4 *Chlamydiaceae* species and 7 strains. Circle 1, genes on forward *Chlamydia trachomatis* strand, color coded by role category; Circle 2, genes on reverse *C. trachomatis* strand; Circle 3, multilocus sequence typing (MLST) candidates, *C. trachomatis*; Circle 4, MLST candidates, *C. pneumoniae* AR39; Circle 5, MLST candidates, *C. caviae* (GPIC); Circle 6, MLST candidates, *C. muridarum* (MoPn). Colors in circles 3, 4, 5 and 6 are consistent for each gene across genomes i.e., "blue" gene in each circle is ortholog in that genome for "blue" gene in *C. trachomatis*. **Blue, *glyA*, serine hydroxymethyl-transferase**; red, tryptophanyl-tRNA synthetase; **yellow, *mdhC*, malate dehydrogenase**; green, V-type ATPase, subunit A; **cyan, *pdhA*, pyruvate dehydrogenase**; black, GTP-binding protein lepa; magenta, transcription termination factor rho; **brown, *yhbG*, probable ABC transporter ATP-binding protein**; **orange, *pykF*, pyruvate kinase**; olive green, conserved hypothetical protein; gray, acetyl-CoA carboxylase beta subunit; pink, threonyl-tRNA synthetase; **violet, *lysS*, lysyl-tRNA synthetase**; **light green, *leuS*, leucyl-tRNA synthetase**. Those denoted in **boldface** above were used for *C. trachomatis* MLST. *ompA* gene location is shown for *C. trachomatis* (dark green).

DNA (GenBank accession nos. FJ45414–FJ746022) using ABI3700 instruments were aligned by using MegAlign (DNASTAR). Each unique sequence for a locus was designated as a unique allele using Sequence Output (www.mlst.net). Each allelic profile (made up of the string of integers corresponding to allele numbers at the 7 loci) was assigned as a different strain or clone and given an ST as a clone descriptor. All STs have been deposited in the *C. trachomatis* site at www.mlst.net.

Allelic profiles and concatenated sequences were used to determine the relatedness of isolates. Average pairwise diversity between isolates was calculated from the 3,714-bp concatenated sequence of the 7 loci for each isolate joined in-frame using MEGA4 (*27*). Synonymous (dS) and nonsynonymous (dN) substitutions were determined using MEGA4 for each locus. Allele frequencies per locus and geographic region were calculated using SAS software 9.2 (SAS Institute, Inc., Cary, NC, USA) with the PROC FREQ tool supplying the frequency count. We calculated a classification index (*11*) on the basis of allele and ST frequency between populations of different geographic regions to determine the probability of association of an allele with a particular continental/subcontinent region. Statistical significance was determined by 10,000 resamplings of allele and ST frequency per region.

Strain Clustering and Single Nucleotide Polymorphism Analyses

eBURST (www.mlst.net) was used to identify clusters of related and singleton STs that were not closely related to any other ST (*12*) and to predict patterns of evolutionary descent. MEGA4 (*27*) was used to construct a tree from concatenated sequences by using minimum evolution, neighbor joining, or unweighted pair group method with arithmetic mean, with various substitution models including Kimura 2-parameter, Jukes Cantor, and p-distance; 1,000 bootstrap replicates were used to test support for each node in the tree. The short evolutionary distances (<≈0.01) imply that back-substitutions were rare, and as expected, all methods gave similar results (data not shown). SplitsTree (www.splitstree.org) was used for evolutionary tree construction by decomposition analyses using the distance matrix produced from pairwise comparisons of concatenated sequences to determine interconnected networks (*28*).

A matrix of all SNPs by ST was produced in Excel. SAS was used to identify which SNPs were associated with an ST using PROC FREQ. Statistical significance was determined by using a classification index as above for the probability of association of a SNP with a particular ST. Levene's test (*29*) was used to determine whether there was equal variance across the 87 isolates. A p value of <0.05 was considered significant.

Results

Discrimination of *C. trachomatis* by MLST

Figure 1 shows genomic alignments for *C. trachomatis* (D/UW-3/CX), *C. muridarum*, *C. pneumoniae* (AR39), and *C. caviae*. The *C. trachomatis* (A/Har-13 and D/UW-3/CX) and *C. pneumoniae* (AR39, CWL029, and J138) genome sequences were almost identical within species for gene content and could be represented by D/UW-3/CX and AR39, respectively.

ompA genotypes were compared with STs resolved by MLST. The online Technical Appendix (available from www.cdc.gov/EID/content/15/9/1385-Techapp.pdf) shows *ompA* genotype (first letter of strain ID), ST, assigned alleles, and clinical characteristics for each isolate. There were 44 STs (0.51 ST/isolate) for the 87 isolates. Thirty STs were represented by a single isolate. In some cases, STI isolates from diverse geographic regions shared the same ST. For example, *ompA* genotype E STI isolates (ST39) were found in California, USA; Amsterdam, the Netherlands; Ecuador; Lisbon, Portugal; and Tanzania. Similarly, LGV genotypes (ST1) were identified in San Francisco, California, USA; Seattle, Washington, USA; and Amsterdam; 2 clinical L_2b

Table 1. Primer pairs used for PCR of chlamydiaceae species and strains

Locus	Region	Primer name	Sequence (5′ → 3′)	Length of sequence, bp
glyA	CT432	FglyA	GAAGACTGTGGCGCTGTTTTATGG	522
		RglyA	CTTCCTGAGCGATCCCTTCTGAC	
mdhC	CT376	FmdhC	GGAGATGTTTTTGGCCTTGATTGT	519
		RmdhC	CGATTACTGCACTACCACGACTCT	
pdhA	CT245	FpdhA	CTACAGAAGCCCGAGTTTTT	549
		RpdhA	CTGTTTGTTGCATGTGGTGATAAG	
yhbG	CT653	FyhbG	TCAAGTCAATGCAGGAGAAAT	504
		RyhbG	GATAGTGTTGACGTACCATAGGAT	
pykF	CT332	FpykF	ATCTTATCGCTGCTTCGTT	525
		RpykF	CAGCAATAATAGGGAGATA	
lysS	CT781	FlysS	GAAGGAATCGATAGAACGCATAAT	576
		RlysS	ATACGCCGCATAACAGGGAAAAAC	
leuS	CT209	FleuS	TCCCTTGGTCGATCTCCTCAC	519
		RleuS	GGGCATCGCAAAAACGTAAATAGT	

genotypes (ST33) were restricted to Amsterdam. In contrast, no trachoma isolates from different continents shared the same ST.

ompA genotypes correlated poorly with relatedness between strains by MLST data (online Technical Appendix). Isolates of the same ST had up to 4 different *ompA* genotypes. For example, ST19 included *ompA* genotypes D, H, I, and J. For each *ompA* genotype, 38%–100% belonged to different STs. Different STs with the same *ompA* genotype were closely related by MLST (e.g., isolates with C and F *ompA* genotypes); others were not. Isolates of D, E, and Ja *ompA* genotypes differed at as many as 5 MLST loci.

Allele Characteristics and Localization by Geography

Allele characteristics are shown in Table 2. The number of alleles at each locus varied from 4 to 11. The average pairwise distance and dS and dN are provided. We determined allele frequencies on the basis of continental/subcontinental regions (Table 3). The majority of alleles were observed multiple times. Seventeen were found only once, and 28 were unique to a specific region (Table 3). The range was from 1 allele at the *lysS* locus for South America to 9 in North America. The highest frequency of a unique allele was 84.6% (*leuS* allele 7) for Asia, which also had the highest proportion of unique alleles, 6/17 (35.29%). There was a significant nonuniform distribution of alleles at each locus by classification index.

Phylogenetic Grouping of STs by Disease Phenotypes and Evidence for Recombination

eBURST (*11*,*12*) generated 3 clonal complexes (CC) (Figure 2): trachoma strains, A, B, Ba, and C (CC-A); noninvasive STIs with low population prevalence (CC-B); and noninvasive, globally prevalent D/Da, E, and F STIs (CC-C). The online Technical Appendix shows eBURST data, including single, double, and triple locus variants (S/D/TLVs).

Relationships between the isolates was further explored by constructing a minimum-evolution tree using MEGA4 (*27*). These data showed 3 disease clusters (Figure 3). Cluster I comprised noninvasive STIs (eBURST CC-B) and a trachoma subcluster (eBURST CC-A). Cluster II comprised only invasive LGV strains. Cluster III included noninvasive prevalent D/Da, E and F STIs (eBURST CC-C). E58t strain (ST39; cluster III) was isolated from the conjunctiva of a trachoma patient, most likely representing autoinoculation from the urogenital tract, because all other isolates of this ST were from STIs.

Nine isolates did not localize on the MLST tree with strains of the same *ompA* genotype (Figure 3). Ja41nl and Ja47nl, which were expected to cluster with other J and Ja isolates in cluster I if the genome sequences were similar, were identical by MLST to reference strain F and clinical isolates F8p, F9p, E19e, and E5s in cluster III. Similarly, D83s, which were expected to cluster with other D and Da isolates in cluster III, had the same ST as H40nl, H18s, I22p, and J44nl in cluster I; D2s were identical to Ia and Ia57e in cluster I. Additionally, G16p did not cluster with the other G isolates in cluster I. In analyzing locations of incongruence between clinical D and E isolates in cluster I, compared with those in cluster III, the loci that differed were *glyA*, *yhbG,* and *pykF* in which allele assignments were identical, in general, to G, H, I, Ia, J, Ja, and K strains (online Technical Appendix) in cluster I. These were the exact same loci that differed for Ja41nl and Ja47nl in cluster III, compared with other J/Ja isolates in cluster I. Ja26s differed at *glyA*, *mdhC*, and *yhbG*, whereas G16p differed at *yhbG, lysS*, and *leuS.* Furthermore, the *ompA* tree (Figure 4) was incongruent with the MLST tree. We interpret all 9 isolates to be recombinants.

SplitsTree decomposition evaluated alternative evolutionary pathways that might indicate recombination between MLST loci (Figure 5). There was considerable network structure, providing evidence of alternative pathways between strains, which may indicate that recombination has influenced the evolution of housekeeping genes for the *C. trachomatis* strains.

SNPs Associated with Disease Phenotypes

We identified 61 polymorphic sites among the 7 loci. Multiple SNPs were significantly associated with each of the 3 clusters and disease groups (Table 4). For example, 15 SNPs in *yhbG* and *leuS* were 100% specific for all LGV strains in cluster III. Any 1 of these SNPs could be used to identify these strains. SNPs 4, 29, 31, 33, and 34 (together or any 1 alone) were specific for the cluster II STIs. For

Table 2. Characteristics of alleles for each locus

Gene locus	No. alleles	Length, bp	No. polymorphic sites	Average pairwise distance	Average dS	Average dN
glyA	7	522	5	0.003	0.0101	0.0034
mdhC	4	519	3	0.001	0.0112	0.0025
pdhA	7	549	6	0.0003	0.0076	0.0030
yhbG	8	504	21	0.01	0.0670	0.0026
pykF	7	525	7	0.003	0.0105	0.0034
lysS	8	576	9	0.002	0.0093	0.0044
leuS	11	519	10	0.003	0.0104	0.0055
Overall	52	3,714	61	0.003		

Table 3. Allele frequencies by geographic region by locus

Gene locus	No. alleles	Allele frequency, no. (%)*						Classification index p value
		Africa (n = 11)	Northern Europe (n = 14)	Southern Europe (n = 10)	Asia (n = 13)	North America (n = 33)	South America (n = 6)	
glyA	7	3 (90.9) 6 (9.1)	1 (7.1) 3 (42.9) **4 (7.1)** **5 (14.3)** 6 (28.6)	3 (60) 6 (40)	3 (30.8) 6 (7.7) **7 (61.5)**	1 (15.1) **2 (3.0)** 3 (54.6) 6 (27.3)	3 (33.3) 6 (66.7)	<0.001
mdhC	4	3 (90.9) 4 (9.1)	1 (7.1) **2 (14.3)** 3 (64.3) 4 (14.3)	3 (80) 4 (20)	3 (100)	1 (18.2) 3 (72.7) 4 (9.1)	3 (50.0) 4 (50.0)	<0.001
pdhA	7	**1 (9.1)** 3 (90.9)	**2 (7.1)** 3 (92.9)	3 (60) **4 (30)** **7 (10)**	3 (100)	3 (94.0) **5 (3.0)** 6 (3.0)	3 (100.0)	<0.001
yhbG	8	2 (9.1) 6 (90.9)	2 (28.6) 3 (7.1) 6 (42.7) 8 (21.4)	2 (40) 6 (50) **7 (10)**	**1 (7.7)** **4 (7.7)** 5 (7.7) 6 (76.9)	2 (21.2) 3 (3.0) 5 (3.0) 6 (57.6) 8 (15.2)	2 (66.7) 6 (33.3)	<0.001
pykF	7	3 (81.8) 6 (9.1) 7 (8.1)	1 (12.5) 6 (50) 7 (37.5)	6 (60) 7 (40)	3 (92.3) 7 (7.7)	1 (18.2) **2 (9.1)** **4 (3.0)** **5 (3.0)** 6 (39.4) 7 (27.3)	6 (33.3) 7 (66.7)	<0.001
lysS	8	4 (15.2) 5 (72.7) **7 (9.1)**	1 (14.3) 4 (78.6) 8 (7.1)	4 (70) 8 (30)	4 (7.7) 5 (30.8) **6 (61.5)**	1 (3.0) **3 (3.0)** 4 (75.8) 8 (18.2)	**2 (16.7)** 4 (66.7) 8 (16.7)	<0.001
leuS	11	2 (9,1) 3 (9.1) 9 (81.8)	3 (57.1) **6 (21.4)** 11(21.4)	3 (80) **4 (10)** **5 (10)**	2 (7.7) **7 (84.6)** **10 (7.7)**	**1 (3.0)** 3 (48.5) **8 (30.3)** 9 (3.0) 11 (15.2)	3 (100.0)	<0.001
Total no. alleles	52	17	24	17	17	31	13	

*Numbers are arranged vertically for each locus to represent the individual alleles (e.g., glyA, alleles are assigned 1, 2, 3, 4, 5, 6 and 7 because there are 7 alleles for this locus). Alleles marked in **boldface** are specific for a single geographic region. n values indicate number of samples.

the trachoma subcluster I, unlike for other clusters, only SNP 38 was associated with reference strains B and C and all clinical trachoma strains; SNPs 54, 55 and 57 together (but not any alone) represented all trachoma strains except reference strain A. Based on classification indices and Levene's test, the null hypothesis of a uniform distribution of SNPs was rejected at the respective locus.

Discussion

Accumulating evidence for recombination among *Chlamydiaceae* in general, and *C. trachomatis* in particular, has motivated a typing system that provides buffering from the distorting effects of genetic reshuffling that plague systems based on a single locus. We therefore developed an MLST scheme derived from comparative genomics of species within the family *Chlamydiaceae* to select conserved chromosomally dispersed housekeeping genes. Our scheme showed considerable variability in allelic profiles associated with geographic regions, as well as diverse and recombinant strains. We also identified SNPs that correlated with the 3 *C. trachomatis* disease groups: invasive LGV diseases, noninvasive urogenital diseases, and trachoma.

Comparative genomics of *Chlamydia* and *Chlamydophila* spp. identified 14 conserved housekeeping genes that could be used to extend MLST schemes for these and potentially other *Chlamydiaceae* spp.. Surprisingly, each gene was located in a different position within the respective genome, indicating a lack of synteny among chromosomes (*20*) (Figure 1), except for the 2 *C. trachomatis* and 3 *C. pneumoniae* strains, which share within species >99% nucleotide sequence identity. This finding suggests that future schemes should select loci to ensure reasonable coverage of the chromosome.

Although there was relatively little sequence diversity in the housekeeping genes, the number of STs (0.51 ST/isolate) was similar to that of other bacterial pathogens. The

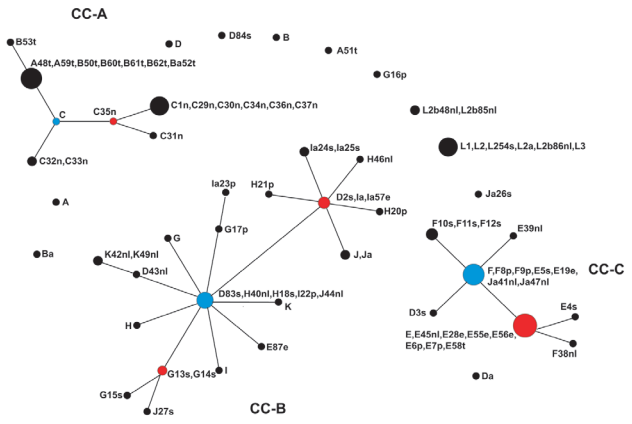

Figure 2. eBURST population snapshot for *Chlamydia trachomatis* isolates. Sequence types (STs) that are linked differ at single multilocus sequence typing locus and represent clonal complexes. Strains in the same clonal complexes are likely descended from the same recent ancestor. Each circle represents a ST. An ST in blue is most likely the primary founder of the clonal complex; STs in red are subgroup founders. The number of isolates of each ST is represented by the area of the circle. Clonal complex A (CC-A) for trachoma strains; CC-B, noninvasive, nonprevalent urogenital strains; and CC-C, noninvasive, globally prevalent urogenital strains. The eBURST report is shown in the online Technical Appendix, available from www.cdc.gov/EID/content/15/9/1385-Techapp.pdf.

previous *C. trachomatis* MLST scheme had 0.60 ST/isolate (*14*). None of the loci were identical to ours. In a recent study of the bacterium *Burkholderia pseudomallei* in Australia, there were 0.65 ST/isolate (*11*,*12*) with relatively little diversity and few alleles per locus. However, high levels of recombination are believed to shuffle alleles to generate different large numbers of allelic profiles (STs). The extent to which recombination among alleles generates novel STs in *C. trachomatis* is unclear. Although the number of STs per isolate varies, the majority of MLST schemes have been successful for strain discrimination, epidemiologic studies, and evaluation of organism evolution (*10*). MLST, however, may not be sufficiently discriminatory for some epidemiologic investigations, even with increased loci numbers. This may be the case for LGV strains, although our scheme resolved 2 L2b strains from all other LGV strains.

We found that a number of STs for STI isolates were shared across continents. This finding was particularly evident for those from Amsterdam, Ecuador, Lisbon, and San Francisco, which would be expected given increasing opportunities for global travel and international sexual encounters. Notably, L2b isolates (ST33) from proctitis cases differed at 2 loci from other LGV isolates (ST1) and were restricted to Amsterdam. Although some L2b strains from Amsterdam and San Francisco have historically been similar (*30*), ST differentiation most likely reflects the emergence of these strains among men who have sex with men. Not surprisingly, STs for trachoma isolates were restricted to the geographic region of origin where populations travel only locally.

Allele frequencies were assigned on the basis of continental/subcontinental regions (Table 3). Most alleles were observed multiple times, and more than half were region specific. Despite the opportunity for worldwide spread, some strains may be stable within the respective geographic populations. This stability was particularly evident in Africa and Asia, where the frequency of unique alleles was the highest, although this finding also reflects the fact that most isolates were from trachoma populations. As expected, we found, in general, a statistically significant nonuniform distribution of alleles.

Analyses using eBURST and trees constructed in MEGA4 resolved isolates into clonal complexes or clusters.

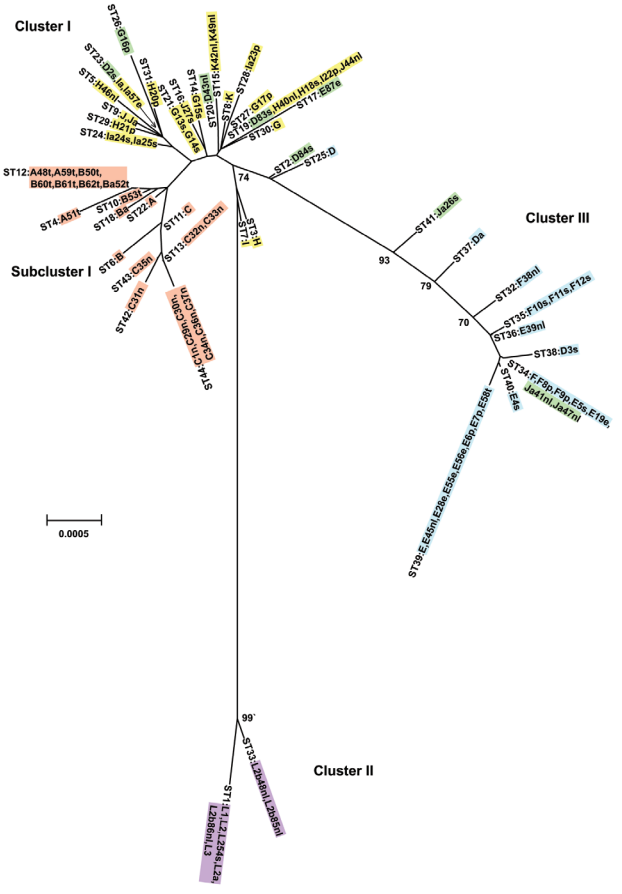

Figure 3. Minimum evolution tree. The tree was constructed using the matrix of pairwise differences between the 87 concatenated sequences for the 7 loci using maximum composite likelihood method for estimating genetic distances. Numbers are bootstrap values (1,000 replicates) >70%. Lavender, invasive lymphogranuloma venereum (LGV); gold, noninvasive, nonprevalent sexually transmitted infection (STI) strains; red, trachoma strains; blue, noninvasive, highly prevalent STI strains; green, putative recombinant stains. Scale bar indicates number of substitutions per site.

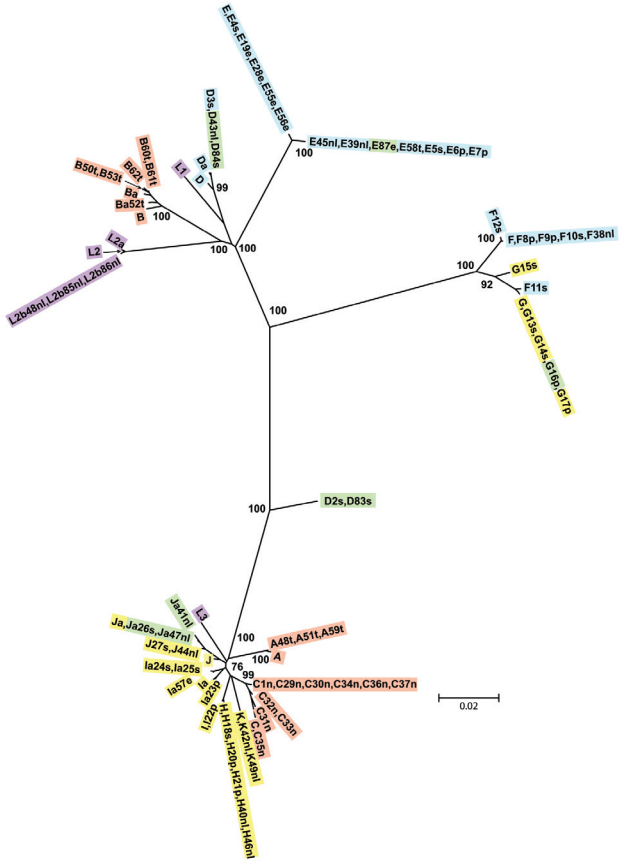

Figure 4. Minimum evolution tree for *ompA*. The tree was constructed using the matrix of pairwise differences between the 87 sequences by using the maximum composite likelihood method for estimating genetic distances. Numbers are bootstrap values (1,000 replicates) >70%. Lavender, invasive lymphogranuloma venereum (LGV); gold, noninvasive, nonprevalent sexually transmitted infection (STI) strains; red, trachoma strains; blue, noninvasive, highly prevalent STI strains; green, putative recombinant stains. Scale bar indicates number of substitutions per site.

Both methods identified distinctive groupings of strains by disease phenotypes. STIs caused by less common strains formed an eBURST group (CC-B) but were within Cluster I on the tree together with the trachoma Subcluster I, which was a separate eBURST group (CC-A). A similar clustering pattern to our tree was found by Pannyhoek et al. (*14*) by using 16 reference and 5 clinical E strains, but they did not distinguish trachoma reference strain B/TW-5 from the LGV group. Our Cluster II included only LGV strains. Cluster III contained the noninvasive globally prevalent D/Da, E, and F strains (eBURST CC-C). This cluster represents efficiently transmitted strains with adaptive fitness in the genital tract.

A number of isolates representing different *ompA* genotypes shared the same ST, whereas many isolates of the same *ompA* genotype had different STs (online Technical Appendix). Furthermore, 9 isolates were found outside the expected cluster, suggesting that recombinational replacement at the *ompA* locus occurs relatively frequently. Accumulating evidence supports frequent recombination among *Chlamydiaceae*. Initial evidence came from observations of recombination within *ompA* (*4*,*31*) followed by phylogenetic analyses (*32*), and bioinformatic and statistical analyses for multiple species of the family Chlamydiaceae and *C. trachomatis* strains (*15*). Recently, we showed intergenic recombination involving *ompA* and *pmpC*, *pmpE-I*, and frequent recombination throughout the genome with significant hotspots for recombination for recent clinical isolates (*16*,*17*). Pannekoek et al. noted incongruence between *ompA* and *fumC* sequences (*14*). Most recombination in our study involved *yhbG, glyA*, and *pykF* (online Appendix Table) with incongruence, compared with *ompA*. Based on *C. trachomatis* recombinants that have been created in vitro, the estimated size of transferred DNA ranged from 123 kb to 790 kb (*33*). Although additional recombination sites may exist in regions that were not sequenced, any gene in our study could be involved in lateral gene exchange with a range of 1,191 bp for a single gene (e.g., *ompA*), 27 kb *(yhbG* to *ompA*) to at least 248 kb (*glyA* to *yhbG*), which is consistent with DeMars and Weinfurter (*33*) and our previous findings (*16*,*17*).

Analysis of the 61 SNPs among the 7 loci showed a statistically significant association of specific polymorphisms with each disease cluster (Table 4). A total of 15 SNPs singly or together identified the LGV cluster. Similarly, 5 SNPs identified the prevalent Cluster II D/Da, E, and F strains. Three clinical D and E strains did not contain these SNPs and each appeared to be a recombinant with other STI strains. Only 1 SNP (in *pykF)* identified all trachoma strains in Subcluster I. Reference trachoma strains A and Ba did not contain this SNP, suggesting that they may not represent circulating strains among present-day populations.

Other studies have associated SNPs or indels in *pmp* and *porB* genes with specific disease causing *C. trachomatis* clades (*16*,*34*,*35*). However, SNPs were not individually analyzed for specific disease associations and the target genes encode surface exposed proteins likely to be under selection for epitope variation to avoid immune system surveillance. A frame-shift mutation in 1 of the tryptophan synthase genes, *trpA*, was associated with trachoma strains when compared with all others, although some B and C strains lack the entire gene (*35*). Large deletions in the cytotoxin loci have also been identified that differentiate the 3 disease groups, yet strain B is missing these loci (*36*). The latter study relied on reference strains, which may limit the use of these deletions for identifying disease-specific groups because clinical isolates may vary in deletion size or location. Additionally, tryptophan synthase genes and cytotoxin loci are located within the 50-kb plasticity zone

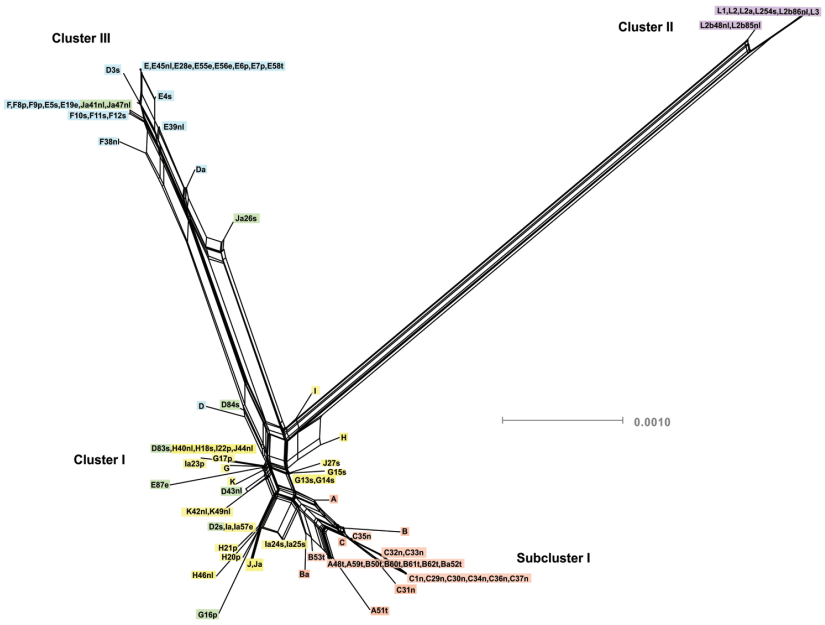

Figure 5. SplitsTree obtained by using concatenated sequences of the 7 loci for the 87 isolates. Cluster I, noninvasive, nonprevalent *Chlamydia trachomatis* strains (gold) with trachoma subcluster I (red); cluster II, invasive lymphogranuloma venereum (LGV) isolates (purple); and cluster III, noninvasive globally prevalent sexually transmitted infection (STI) strains (blue). Isolates colored green represent putative recombinant strains. Scale bar indicates number of substitutions per site.

of the chromosome, a region known for genetic reshuffling (*20*). The current study differs from those previously mentioned in that it used housekeeping genes that are not under immune selection or in the plasticity zone. Therefore, the SNPs we identified are probably neutral and can be used as reliable markers for disease association. Furthermore, SNPs were based on reference and clinical isolates of multiples of the same strains from 6 geographic regions, representing a broad diversity of this species.

Table 4. SNPs and combinations of SNPs that correlate 100% with designated cluster and disease phenotype group*

Gene locus	SNP no.	Cluster III	Cluster II	Subcluster I†
glyA	1–5		4‡	
mdhC	6–8			
pdhA	9–14			
yhbG	15–35	15§	29‡	
		20	31	
		22–26	33	
		30	34	
pykF	36–42			38¶
lysS	43–51			
leuS	52–61	52§		54
		56		55
		61		57
Total no. SNPs	61	15	5	4

*SNP, single nucleotide polymorphism. Each SNP is 100% specific for designated cluster and disease group (e.g., SNP 15 identifies cluster III but all 15 SNPs are specific to cluster III). Cluster III comprises all clinical and reference lymphogranuloma venereum (LGV) strains; cluster II comprises all reference and clinical D, Da, E, F, and recombinant clinical Ja strains except recombinant clinical D2s, D43nl, E87e, and reference Ja strains; subcluster I comprises all reference and clinical trachoma A, B, Ba and C strains except reference strains A and Ba. Disease phenotype groups: invasive LGV strains (cluster III); trachoma A, B, Ba and C strains (subcluster I); and noninvasive globally prevalent STI D/Da, E, and F strains (cluster II). Clinical Ja strains likely acquired a Ja *ompA* gene by recombination.
†SNPs 54, 55, and 57 together (not independently) are specific for all trachoma strains except reference A strain.
‡p<0.01 for classification index.
§p<0.001 for classification index.
¶p = 0.008 for classification index.

Given the high rates of infection among STI (*37*) and trachoma populations (*38*,*39*), the ability to distinguish LGV and noninvasive urogenital and trachoma strains, including mixed infections, would aid epidemiologists, clinicians, and public healthcare workers worldwide in determining appropriate therapeutic or intervention strategies (*40*). Our multilocus and SNP typing can now be used to standardize the way an organism is typed; isolates from diverse geographic regions worldwide can be identified and compared; and diverse and emerging *C. trachomatis* strains can be detected for epidemiologic and evolutionary studies among trachoma and STI populations worldwide.

Acknowledgments

We thank Hugh Taylor for providing trachoma samples from Tanzania.

This work was supported by NIH grants R01 AI059647, R01 AI039499 and R01 EY/AI012219 (D.D.), a European Union grant EU-FP6-LSHG-CT-2007-037637 (S.A.M.), and a Wellcome Trust Grant 030662 (B.G.S.).

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References

1. Wang SP, Grayston JT. Three new serovars of *Chlamydia trachomatis*: Da, Ia, and L2a. J Infect Dis. 1991;163:403–5.
2. Dean D, Schachter J, Dawson CR, Stephens RS. Comparison of the major outer membrane protein variant sequence regions of B/Ba isolates: a molecular epidemiologic approach to *Chlamydia trachomatis* infections. J Infect Dis. 1992;166:383–92.
3. Hayes LJ, Bailey RL, Mabey DC, Clarke IN, Pickett MA, Watt PJ, et al. Genotyping of *Chlamydia trachomatis* from a trachoma-endemic village in the Gambia by a nested polymerase chain reaction: identification of strain variants. J Infect Dis. 1992;166:1173–7.
4. Brunham R, Yang C, Maclean I, Kimani J, Maitha G, Plummer F. *Chlamydia trachomatis* from individuals in a sexually transmitted diseases core group exhibit frequent sequence variation in the major outer membrane protein (*omp1*) gene. J Clin Invest. 1994;94:458–63. DOI: 10.1172/JCI117347
5. Dean D, Oudens E, Bolan G, Padian N, Schachter J. Major outer membrane protein variants of *Chlamydia trachomatis* are associated with severe upper genital tract infections and histopathology in San Francisco. J Infect Dis. 1995;172:1013–22.
6. Hamill M, Benn P, Carder C, Copas A, Ward H, Ison C, et al. The clinical manifestations of anorectal infection with lymphogranuloma venereum (LGV) versus non-LGV strains of *Chlamydia trachomatis*: a case-control study in homosexual men. Int J STD AIDS. 2007;18:472–5. DOI: 10.1258/095646207781147319
7. Frost EH, Deslandes S, Veilleux S, Bourgaux-Ramoisy D. Typing *Chlamydia trachomatis* by detection of restriction fragment length polymorphism in the gene encoding the major outer membrane protein. J Infect Dis. 1991;163:1103–7.
8. Rodriguez P, Allardet-Servent A, de Barbeyrac B, Ramuz M, Bebear C. Genetic variability among *Chlamydia trachomatis* reference and clinical strains analyzed by pulsed-field gel electrophoresis. J Clin Microbiol. 1994;32:2921–8.
9. Morré SA, Ossewaarde JM, Savelkoul PH, Stoof J, Meijer CJ, van den Brule AJ. Analysis of genetic heterogeneity in *Chlamydia trachomatis* clinical isolates of serovars D, E, and F by amplified fragment length polymorphism. J Clin Microbiol. 2000;38:3463–6.
10. Maiden MC. Multilocus sequence typing of bacteria. Annu Rev Microbiol. 2006;60:561–88. DOI: 10.1146/annurev.micro.59.030804.121325
11. Jolley KA, Wilson DJ, Kriz P, McVean G, Maiden MC. The influence of mutation, recombination, population history, and selection on patterns of genetic diversity in *Neisseria meningitidis*. Mol Biol Evol. 2005;22:562–9. DOI: 10.1093/molbev/msi041
12. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186:1518–30. DOI: 10.1128/JB.186.5.1518-1530.2004
13. Klint M, Fuxelius HH, Goldkuhl RR, Skarin H, Rutemark C, Andersson SG, et al. High-resolution genotyping of *Chlamydia trachomatis* strains by multilocus sequence analysis. J Clin Microbiol. 2007;45:1410–4. DOI: 10.1128/JCM.02301-06
14. Pannekoek Y, Morelli G, Kusecek B, Morre SA, Ossewaarde JM, Langerak AA, et al. Multi locus sequence typing of Chlamydiales: clonal groupings within the obligate intracellular bacteria *Chlamydia trachomatis*. BMC Microbiol. 2008;8:42. DOI: 10.1186/1471-2180-8-42
15. Millman KL, Tavare S, Dean D. Recombination in the *ompA* gene but not the *omcB* gene of *Chlamydia* contributes to serovar-specific differences in tissue tropism, immune surveillance, and persistence of the organism. J Bacteriol. 2001;183:5997–6008. DOI: 10.1128/JB.183.20.5997-6008.2001
16. Gomes JP, Nunes A, Bruno WJ, Borrego MJ, Florindo C, Dean D. Polymorphisms in the nine polymorphic membrane proteins of *Chlamydia trachomatis* across all serovars: evidence for serovar Da recombination and correlation with tissue tropism. J Bacteriol. 2006;188:275–86. DOI: 10.1128/JB.188.1.275-286.2006
17. Gomes JP, Bruno WJ, Nunes A, Santos N, Florindo C, Borrego MJ, et al. Evolution of *Chlamydia trachomatis* diversity occurs by widespread interstrain recombination involving hotspots. Genome Res. 2007;17:50–60. DOI: 10.1101/gr.5674706
18. Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, et al. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis.* Science. 1998;282:754–9. DOI: 10.1126/science.282.5389.754
19. Carlson JH, Porcella SF, McClarty G, Caldwell HD. Comparative genomic analysis of *Chlamydia trachomatis* oculotropic and genitotropic strains. Infect Immun. 2005;73:6407–18. DOI: 10.1128/IAI.73.10.6407-6418.2005
20. Read TD, Brunham RC, Shen C, Gill SR, Heidelberg JF, White O, et al. Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. Nucleic Acids Res. 2000;28:1397–406. DOI: 10.1093/nar/28.6.1397
21. Shirai M, Hirakawa H, Kimoto M, Tabuchi M, Kishi F, Ouchi K, et al. Comparison of whole genome sequences of *Chlamydia pneumoniae* J138 from Japan and CWL029 from USA. Nucleic Acids Res. 2000;28:2311–4. DOI: 10.1093/nar/28.12.2311
22. Read TD, Myers GS, Brunham RC, Nelson WC, Paulsen IT, Heidelberg J, et al. Genome sequence of *Chlamydophila caviae* (*Chlamydia psittaci* GPIC): examining the role of niche-specific genes in the evolution of the *Chlamydiaceae*. Nucleic Acids Res. 2003;31:2134–47. DOI: 10.1093/nar/gkg321
23. Grant JR, Stothard P. The CGView Server: a comparative genomics tool for circular genomes. Nucleic Acids Res. 2008;36:W181-4. DOI: 10.1093/nar/gkn179
24. Rasko DA, Myers GS, Ravel J. Visualization of comparative genomic analyses by BLAST score ratio. BMC Bioinformatics. 2005;6:2. DOI: 10.1186/1471-2105-6-2
25. Millman K, Black CM, Johnson RE, Stamm WE, Jones RB, Hook EW, et al. Population-based genetic and evolutionary analysis of *Chlamydia trachomatis* urogenital strain variation in the United States. J Bacteriol. 2004;186:2457–65. DOI: 10.1128/JB.186.8.2457-2465.2004
26. Meats E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS, et al. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol. 2003;41:1623–36. DOI: 10.1128/JCM.41.4.1623-1636.2003
27. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
28. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006;23:254–67. DOI: 10.1093/molbev/msj030
29. Levene H. Robust tests for the equality of variances. In: Olkin I, editor. Contributions to probability and statistics: essays in honor of Harold Hotelling. Stanford (CA): Stanford University Press; 1960. p. 278–92.
30. Spaargaren J, Schachter J, Moncada J, de Vries HJ, Fennema HS, Pena AS, et al. Slow epidemic of lymphogranuloma venereum L2b strain. Emerg Infect Dis. 2005;11:1787–8.
31. Hayes LJ, Yearsley P, Treharne JD, Ballard RA, Fehler GH, Ward ME. Evidence for naturally occurring recombination in the gene encoding the major outer membrane protein of lymphogranuloma venereum isolates of *Chlamydia trachomatis*. Infect Immun. 1994;62:5659–63.

32. Fitch WM, Peterson EM, de la Maza LM. Phylogenetic analysis of the outer-membrane-protein genes of chlamydiae, and its implication for vaccine development. Mol Biol Evol. 1993;10:892–913.
33. DeMars R, Weinfurter J. Interstrain gene transfer in *Chlamydia trachomatis* in vitro: mechanism and significance. J Bacteriol. 2008;190:1605–14. DOI: 10.1128/JB.01592-07
34. Brunelle BW, Sensabaugh GF. The *ompA* gene in *Chlamydia trachomatis* differs in phylogeny and rate of evolution from other regions of the genome. Infect Immun. 2006;74:578–85. DOI: 10.1128/IAI.74.1.578-585.2006
35. Caldwell HD, Wood H, Crane D, Bailey R, Jones RB, Mabey D, et al. Polymorphisms in *Chlamydia trachomatis* tryptophan synthase genes differentiate between genital and ocular isolates. J Clin Invest. 2003;111:1757–69.
36. Carlson JH, Hughes S, Hogan D, Cieplak G, Sturdevant DE, McClarty G, et al. Polymorphisms in the *Chlamydia trachomatis* cytotoxin locus associated with ocular and genital isolates. Infect Immun. 2004;72:7063–72. DOI: 10.1128/IAI.72.12.7063-7072.2004
37. Fine D, Dicker L, Mosure D, Berman S. Increasing chlamydia positivity in women screened in family planning clinics: do we know why? Sex Transm Dis. 2008;35:47–52. DOI: 10.1097/OLQ.0b013e31813e0c26
38. Dean D, Kandel RP, Adhikari HK, Hessel T. Multiple *Chlamydiaceae* species in trachoma: implications for disease pathogenesis and control. PLoS Med. 2008;5:e14. DOI: 10.1371/journal.pmed.0050014
39. Somboonna N, Mead S, Liu J, Dean D. Discovering and differentiating new and emerging clonal populations of *Chlamydia trachomatis* with a novel shotgun cell culture harvest assay. Emerg Infect Dis. 2008;14:445–53. DOI: 10.3201/eid1403.071071
40. McLean CA, Stoner BP, Workowski KA. Treatment of lymphogranuloma venereum. Clin Infect Dis. 2007;44(Suppl 3):S147–52. DOI: 10.1086/511427

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Increasing Incidence of Zygomycosis (Mucormycosis), France, 1997–2006

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We analyzed hospital records to provide a population-based estimate of zygomycosis incidence and trends over a 10-year period at a national level in France. Data showed an increasing incidence from 0.7/million in 1997 to 1.2/million in 2006 ($p<0.001$). We compared our data with those from the French Mycosis Study Group, a recently established voluntary network of French mycologists coordinated by the National Reference Center for Mycoses and Antifungals. We documented that incidence of zygomycosis increased, particularly in patients with hematologic malignancies or bone marrow transplants. The role of previous exposure to antifungal drugs lacking activity against zygomycetes could explain this increase but does not appear exclusive. Incidence also increased in the population of patients with diabetes mellitus. We conclude that observed trends reflect a genuine increase of zygomycosis cases in at-risk populations.

Zygomycoses are severe angioinvasive infections caused by common filamentous fungi, the zygomycetes. These ubiquitous opportunistic fungi can cause infections with high lethality in immunocompromised or diabetic patients. Whatever the route of infection (inhalation of airborne spores, ingestion, or direct skin inoculation), the hyphae invade blood vessels, causing tissue infarction and necrosis (*1–4*). In healthy persons, innate immunity is sufficient to prevent infection, except in cases of massive contamination after traumatic inoculation of contaminated soil (*5*). Patients with phagocytic dysfunctions caused by neutropenia or ketoacidosis, as well as patients with high iron serum concentrations, are at high risk of developing zygomycosis (*4*). These underlying conditions can influence clinical presentation and outcome (*2*,*6*,*7*). The rhinocerebral presentation is the most frequently reported localized symptom followed by pulmonary, cutaneous, cerebral, gastrointestinal, and disseminated infections (*3*,*8*). In the rhinocerebral or pulmonary forms, patient death rates are reported to be as high as 60% because of delayed diagnosis or delayed therapeutic management (*7–9*). Treatment strategies are based on high doses of any lipid formulation of amphotericin B, associated with large surgical resections when possible. Most triazole agents are not effective in vivo (*10*), except for posaconazole, which shows some efficacy both experimentally and in patients as second-line therapy (*11–13*). The clinical contribution of new iron chelating agents remains controversial (*14*,*15*).

Some reports have suggested an increasing incidence of zygomycosis on the basis of analysis of data from monocentric studies (*7*,*10*,*14*,*16*). Several explanations have been posited: longer survival of persons with severe hematologic malignancies or solid organ transplantations; increased knowledge of the infection and thus a better diagnosis yield; and finally, prolonged use of new antifungal drugs ineffective against zygomycetes as prophylactic or empiric treatment in bone marrow transplant recipients or other immunocompromised patients (*10*,*17–20*). However, the incidence of infection among the general population and infection trends over time has rarely been documented at a national level. To describe the demographic characteristics of patients and to estimate the incidence and case-fatality ratio (CFR) associated with the major underlying diseases, we analyzed the electronic hospitalization and death records of patients in France in whom zygomycosis had been diagnosed.

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DOI: 10.3201/eid1509.090334

Materials and Methods

Data Sources

To describe cases and to estimate incidence, we extracted all hospital records related to zygomycosis from 1997 through 2006 in metropolitan France, which includes mainland France and the island of Corsica. The French hospital information system, Programme de Médicalisation du Système d'Information (PMSI), is a managerial tool based on the systematic collection of standardized administrative and medical information for any new hospital admission. An estimated 95% of all public and private hospitals use this tool, including all third-level structures, i.e., university hospitals and other reference hospitals (*21*). An anonymous extraction of the dataset with limited sociodemographic information (age, sex, and residence area) can be made available for specific epidemiologic studies. Medical information includes the main cause of admission (principal diagnosis), the related medical conditions (with >20 entries for various associated diagnoses), duration of stay, modes of admission and discharge, and major medical activities performed during the stay. Diseases are coded according to the International Classification of Diseases, 10th revision (ICD-10). This coding system was stable during the study period. Conversely, the codes for medical or surgical procedures, determined at the national level, have evolved over time, with notable changes occurring in 2003.

To estimate the overall CFR, deaths that occurred during hospitalization, including those among readmitted patients, were identified through the PMSI. To take into account deaths that occurred after discharge, we also used data from death certificates available at the institution in charge of recording and analyzing death certificates, the CepiDc. The certificate is completed at the time of death by the attending physician (at hospital or home) who records the main cause of death (i.e., the direct, immediate cause), as well as underlying diseases that may have contributed to death. The certificate is then sent to the CepiDc, where it is coded according to ICD-10.

Case Identification

We used all PMSI records in which the ICD-10 codes B46 or B460 to B469 were identified; these corresponded to any clinical presentation of zygomycosis, or mucormycosis. To distinguish first admissions from rehospitalizations, we created a unique patient identifier by chaining the variables year of birth (derived from the patient's age and the date of admission), gender, and residence postal code. The same identifier was used to match PMSI and CepiDc data, to identify additional deaths and to check for duplicates. However, the CepiDc identifier was based on the exact year of birth, but in the PMSI we derived the year of birth from age at admission.

Case Definitions

We defined as unique cases those that had an identifier detected only 1 time in the PMSI during the 10-year period. Cases detected >1 time were classified as new cases at first occurrence in the database; subsequent ones were coded as readmissions. After checking for duplicates, we analyzed unique or new cases for incidence.

The zygomycosis cases were classified according to their clinical presentation, when available. When several body localizations were reported, we selected the most severe one, either in a single stay or in subsequent admissions. For instance, the association of unspecified localization (B46, B468, or B469) or gastrointestinal (B462) or cutaneous (B463) localizations, together with a pulmonary (B461), a rhinocerebral (B462), or a disseminated (B464) localization, was classified under 1 of the 3 latter categories.

We similarly analyzed the underlying diseases potentially associated with zygomycosis and focused on known risk factors. We combined the ICD-10 codes with medical procedures because some conditions, such as bone marrow transplantation, were coded as a procedure and not as a disease. To take into consideration patients with >1 underlying condition, we introduced a hierarchy. We first created the bone marrow transplantation (BMT) category, which included autologous and allogenic BMT, with or without graft versus host disease. To reduce underreporting bias, we looked for BMT occurrence in both incident and readmitted patients. Because of changes in the coding system regarding medical procedures, identification of autologous versus allogenic BMTs before 2003 was not possible. In a second category labeled hematologic malignancies, we included neutropenia or aplastic anemia, acute lymphoid or myeloid leukemia, lymphomas, and other disorders of the myeloid system. The third category, labeled nonhematologic immunodepression, included solid organ cancers, HIV infection, solid organ transplantations, and/or rejection of these transplantations. By convention, BMT patients (with or without graft versus host disease) who also had a neutropenia were classified under the BMT category. Finally, we analyzed diabetes types 1 or 2 patients under a separate category; for patients who had both an immunosuppressive condition and diabetes, the immunosuppressive condition was kept as the main risk factor.

Data Analysis

Taking into account population growth, we used the 1999 national population census to estimate annual incidence rates. To enable trends comparisons, we used the same approach for the specific incidence rates in patients with underlying factors (zygomycosis patients presenting these underlying factors in the numerator and total population in the denominator). Because of heterogeneous age

distributions in the population according to geographic areas, standardization of incidence rates by gender and age groups was performed in each of the 96 districts (departments) of metropolitan France. Populations in the districts ranged from 73,500 to >2.5 million inhabitants.

For CFR, we considered a possible underreporting in the PMSI combined with inaccuracies in the registration of causes of deaths in the CepiDc. To estimate the number of deaths that could have been missed by both data sources, we performed a capture–recapture analysis following the method synthesized by Gallay et al. (*22*). In a 2 × 2 table, the numbers of deaths identified in one of the respective databases ($n_{1,2}$ or $n_{2,1}$, in which the subscript 1 indicates identified and 2 indicates not identified) or in both databases ($n_{1,1}$) are entered in the respective 2 × 2 table's boxes. Under the hypothesis that both sources are independent, deaths that could have been missed by both sources ($n_{2,2}$) can be estimated by the equation ($n_{2,2} = n_{1,2} \times n_{2,1} / n_{1,1}$). The total number of deaths is $N = n_{1,1} + n_{1,2} + n_{2,1} + n_{2,2}$. The variance and 95% confidence intervals (CIs) are $Var(N) = (n_{1,1} + n_{2,1})(n_{1,1} + n_{1,2})(n_{1,2})(n_{2,1})/(n_{1,1})^3$ and 95% CI = $N \pm 1.96Var(N)$, respectively.

The CFR estimates were impaired by the unavailability of accurate information about underlying medical conditions for deaths identified in the CepiDc. In addition, the approximation of the year of birth when using the PMSI dataset limited the CFR studies by age groups. We therefore restricted the analysis to the overall CFR by underlying diseases whenever possible and made no further assumptions on missing data.

Statistical Analysis

Statistical analysis was performed by using Excel (Microsoft Corporation, Redmond,, WA, USA) and STATA-9 (StataCorp LP, College Station, TX, USA) software. The Fisher exact or χ^2 tests were used where appropriate to compare groups. Trends were assessed by using a Poisson regression. Where appropriate, we present results with 95% CIs and p values, considering $p \leq 0.05$ as significant.

Results

Increased Incidence of Zygomycosis in Metropolitan France

Of 828 hospital stays linked to zygomycosis, 531 incident cases were identified in public and private hospitals of metropolitan France from 1997 through 2006 There were 283 males and 248 females (sex ratio 1.1); mean age was 57.1 years (median 60 years, range: <1 month–96 years). The annual incidence rate (AIR) increased from 0.7 cases/million persons in 1997 to 1.2/million persons in 2006 (Figure 1); yearly increase was +7.4% (p<0.001). Averaged over the 10 years of study, the AIR was 0.9/million persons (95% CI 0.8–1.0). This average AIR increased with age from 0.3/million in children aged 0–9 years to 3.9/million in patients >89 years of age (Figure 2).

The number of patients per district of residence varied from 0 to 36 over the 10-year period. The average standardized incidence rate varied from 0 to 3.4/million persons per district per year. The highest rates were observed in 5 rural districts hosting <500,000 inhabitants each (p<0.01): from 2.4/million (95% CI 2.3–2.5) to 3.4/million (95% CI 3.2–3.5) versus 0.9/million nationwide. In the greater Paris area hosting ≈10 million inhabitants in 8 districts, the average standardized incidence rate was 1.1/million per year (95% CI 0.9–1.2), not significantly different from the rate for the nation.

Underlying diseases among the 531 zygomycosis case-patients comprised 156 patients with immunosuppressive conditions: 33 patients with BMT, 59 patients with hematologic malignancies, and 64 patients with nonhematologic immunodepression (Table). Of the 104 patients with diabetes, 17 also experienced an underlying immunosuppressive condition and were classified as such. In zygomycosis patients with hematologic malignancies, the specific AIR increased over time from 0.02 to 0.2 cases/million persons from 1997 through 2006 (+24%/year; p<0.001) (Figure 1). It also increased in patients with BMT (+15%/year; p = 0.02) or with diabetes (+9%/year; p = 0.02). Incidence was not significantly altered over time for patients with nonhematologic immunodepression and in patients with no known risk factors (data not shown).

Clinical Presentation

The reported presentations included pulmonary (18.5%), rhinocerebral (11.8%), cutaneous (9.4%), and disseminated (6.8%) localizations. The digestive tract was mentioned as a unique localization for 123 (23.2%) patients. No information was available for 30.3% of patients.

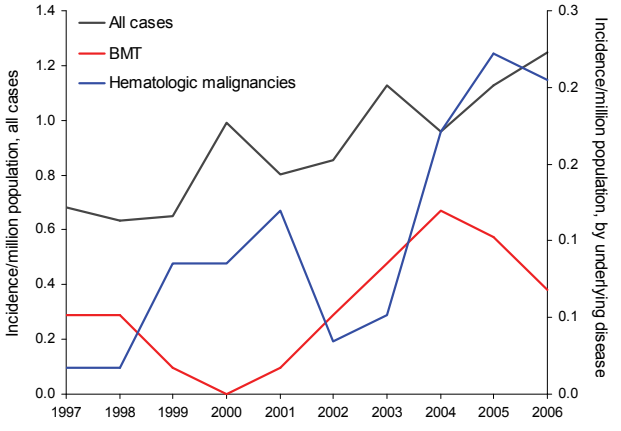

Figure 1. Evolution of the incidence of zygomycosis, France, 1997–2006. BMT, bone marrow transplantation.

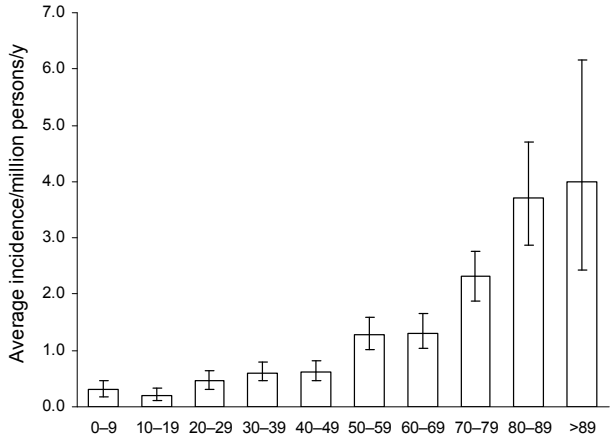

Figure 2. Average annual incidence rate of zygomycosis, by age group, France, 1997–2006. Error bars indicate 95% confidence intervals.

Deaths

Sixty-one deaths were recorded in the PMSI dataset, including 7 deaths reported among readmitted patients. We identified 31 additional deaths in the CepiDc, resulting in a total of 92 deaths during the study period and an overall CFR of 17.3% (95% CI 9.1–31.5). Lethality evolved over time and increased from 2004 onward: average CFR was 13.7% in the 1997–2003 period versus 23.6% in the 2004–2006 period ($p<0.01$). Using the capture–recapture method, the overall expected number of deaths would reach 143 (95% CI 0–40) for a CFR of 26.9% (95% CI 0–100%). The specific CFR according to underlying conditions was calculated only for the 61 deaths reported in the PMSI for which information was available (Table). It was greater when patients had hematologic malignancies (47.8%) or BMT (36.4%). In patients with nonhematologic immunodepression, diabetes, or no known risk factors, the CFRs were 12.5%, 9.3%, and 4.2%, respectively.

Discussion

Incidence and Trends

This retrospective analysis of hospital records provides a population-based estimate of zygomycosis incidence and trends over a 10-year period at a national level. It shows an increasing incidence from 0.7/million to 1.2/million population ($p<0.001$). The average 0.9/million annual incidence rate in France is lower than the 1.7/million incidence reported in the population-based study by Rees et al. in California in 1992–1993 (*23*). However, our results are based on a passive routine system, whereas an active laboratory-based surveillance was implemented in the San Francisco Bay area at a time of high HIV prevalence; this system had smaller sample sizes (2.9 million in San Francisco vs. ≈60 million in France). In a survey performed in Spain in 2005, incidence was $0.43/10^6$ on a representative sample of 50 participating hospitals covering one third of the country's population, i.e., ≈14 million inhabitants (*24*).

We compared our data with those from the French Mycosis Study Group, a recently established voluntary network of French mycologists coordinated by the National Reference Center for Mycoses and Antifungals (NRCMA). This network, which covers all university and other tertiary level hospitals in France, reported an average of 17 cases per year since 2003 versus 65 in our study for the same period. These differences could have several nonexclusive reasons. First, diagnosing zygomycosis is not easy. As recently underlined (*25*), a culture positive with a zygomycete does not always mean infection; many gastrointestinal cases reported in the PMSI may be false-positive cases resulting from laboratory contamination. The unusual distribution of clinical presentations found in our study and the fact that one third of clinical information was missing favor this hypothesis. Second, we may have overestimated the number of cases because of undetected duplicates or coding errors. However, inversely, underreporting from the voluntary-based NRCMA network may also occur.

The relevance of hospital discharge data based on ICD-10 codes to estimate the incidence of a rare disease is regularly debated because of limitations in diagnosis accuracy, inconsistent disease coding, or undetected duplicates because of anonymous data recording (*26*). As reported by Chang et al. (*27*), who compared administrative data with medical records in the United States, the positive predictive value of ICD-10 codes for detecting invasive aspergillosis can be low. The authors stressed that these codes may sometimes be assigned before final diagnosis is confirmed, or they may not be assigned by the clinician in charge of the patient. These considerations are also true in the PMSI: overreporting was estimated at 11% for cancers of the thyroid in a study based in 10 districts (*28*) and at 15% for mesothelioma (*29*). Inversely, underreporting caused by diagnostic or coding errors was estimated at 17% by Hafdi-Nejjari et al. (*30*) and at 27% by Carré et al. (*28*), respectively. Nevertheless, administrative data are useful tools to estimate the trends of severe infections like zygomycosis, since most patients are likely to be hospitalized (*26,31*), especially in the tertiary care centers that participate in the PMSI.

We were able to document that the incidence of zygomycosis increased in the population of patients with hematologic malignancies or BMT, as already noticed in the 1990s (*32*). In this population, increases in the number of recorded cases could be linked to frequent prescription of antifungal drugs lacking activity against zygomycetes, such as voriconazole and caspofungin (*10,17,18,33–35*).

Table. Distribution of zygomycosis cases and deaths, by underlying disease, France, 1997–2006

Known risk factors	No. cases	No. deaths	Case-fatality ratio, %*
Bone marrow transplantation	33	12	36.4
Hematologic malignancies	59	21	35.6
Neutropenia/aplastic anemia	23	11	47.8
Acute lymphoid/myeloid leukemias	20	6	30.0
Other lymphoid/myeloid disorders	16	4	25.0
Nonhematologic immunodepression	64	8	12.5
Cancers of the solid organs	28	3	10.7
HIV/AIDS	26	1	3.8
Solid organ transplantations	10	4	40.0
Diabetes	86	8	9.3
No known risk factor	289	12	4.2
Total	531	61	11.5

*Calculated only for the 61 deaths reported in hospital records for which information about underlying diseases was available.

However, the role of previous exposure to these antifungal drugs does not appear exclusive because the incidence also increased in the population of patients with diabetes mellitus, a population not known to be commonly exposed to long-term antifungal therapies. An increasing number of the populations at risk (increasing incidence or prolonged survival times) could explain the increase observed in our study: +24% per year for patients with hematologic malignancies and +15% for BMT patients, respectively. However, data from the national agency in charge of the surveillance of transplantations (*36*) indicate that the number of hematopoietic stem cell transplantations increased by +1.58% per year ($p<0.01$) 2001–2006. Similarly, we documented a +9% per year increase in zygomycosis in patients with diabetes; in parallel, diabetes prevalence increased at an estimated 5.7%/year in France from 2000 through 2005 (*37*). We thus tend to consider that the observed trends reflect a genuine increase of zygomycosis cases in these at-risk populations.

Patients' Characteristics

The PMSI provides scarce information about the sociodemographic characteristics of patients. In a review of 929 cases published from 1940 through 1999, Roden et al. (*8*) indicated a 1.8 M:F sex ratio and an average age of 38.8 years, versus 1.1 M:F sex ratio and 57.1 years in our study, respectively. However, age distributions and underlying conditions are likely to differ between places and to evolve over time, thus not allowing strict comparisons. Another limitation of the PMSI is the absence of information regarding the circumstances of exposure and portal of entry. The highest incidence rates found in 5 rural districts and not in others of comparable age and gender distributions may indicate a particular exposure to molds in rural environments (for instance, a professional exposure) or an increased awareness of these diseases among clinicians and microbiologists/mycologists of the corresponding regions.

Patient CFRs

Reporting biases may also partially explain the overall low CFR in our study: 26.9% in our highest estimate versus 54% in Roden's review of 929 cases (*8*). Nevertheless, the CFRs for patients with severe immunosuppressive conditions (Table) are close to values reported in the literature. The CFRs in our study are population-based, while reports from the literature mostly refer to large but relatively old series or to series of immunocompromised patients with severe underlying diseases whose outcome is expected to be worse than in the nonimmunocompromised general population (*8*,*32*,*38*). Also, the CFRs in these other studies may include the long-term outcomes for more discrete time frames, for example, 1 year survival rates (*32*), information not available through our type of study. Deaths occurring during hospitalization are not systematically reported in the PMSI, and those occurring outside the hospital may be underdiagnosed in the CepiDc because many practitioners are not aware of zygomycosis. False-positive cases (for instance, zygomycetes culture without infection) can also influence CFR calculations by increasing the denominator. The wide range of CFR estimates calculated with the capture–recapture method reflects these uncertainties.

Recent progress in early diagnosis and treatment strategies over time should also be considered. For instance, the CFR of rhinocerebral forms may vary from 20% to 69% (*16,39*). Roden et al. showed a decrease of the global CFR from 84% to 47% over time since the 1950s (*8*). Antifungal drugs combined with surgical resections play an important role in decreasing lethality for localized infections. The CFR reportedly decreased from 70% in patients treated with antifungal drugs alone down to 14% in patients who receive combined antifungal drugs and surgical treatment (*40*).

Conclusion

Given the stable increase of incidence over years especially among patients presenting known risk factors

(Figure 1), we tend to consider that the increasing numbers of zygomycosis cases are likely to reflect the actual temporal trends of zygomycosis infections diagnosed in the French public and private hospitals. Despite the limitations linked to the sources of data we used, this study provides a useful overview of the zygomycosis incidence trends over a decade at a western European country level. It stresses the growing frequency of this severe infection and the need to increase awareness among clinicians of early diagnosis and treatment, especially for patients with hematologic malignancies, BMT, and/or diabetes. As stated by Chang et al. (*27*), administrative data can be regarded as useful screening tools that should be completed by medical record investigation. In fact, our study has represented an opportunity to launch in 2008 a retrospective study of zygomycosis cases identified through the PMSI and the NRCMA. This study will provide a description of zygomycosis cases and their outcome in France. In addition, it should enable us to evaluate the predictive value of administrative data for this rare disease.

Acknowledgments

We thank Javier Nicolau and Pascale Bernillon for their support with data extraction and analysis. We also thank Serge Alfandari and Jean Michel Thiolet for their helpful review of the manuscript.

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References

1. Dromer F, Mc Ginnis MR. Zygomycosis. In: Anaissie EJ, Mc Ginnis MR, Pfaller MA, editors. Clinical mycology. New York: Churchill Livingstone; 2002. p. 297–308.
2. Greenberg RN, Scott LJ, Vaughn HH, Ribes JA. Zygomycosis (mucormycosis): emerging clinical importance and new treatments. Curr Opin Infect Dis. 2004;17:517–25. DOI: 10.1097/00001432-200412000-00003
3. Ribes JA, Vanover-Sams CL, Baker DJ. Zygomycetes in human disease. Clin Microbiol Rev. 2000;13:236–301. DOI: 10.1128/CMR.13.2.236-301.2000
4. Spellberg B, Edwards J Jr, Ibrahim A. Novel perspectives on mucormycosis: pathophysiology, presentation, and management. Clin Microbiol Rev. 2005;18:556–69. DOI: 10.1128/CMR.18.3.556-569.2005
5. Liang KP, Tleyjeh IM, Wilson WR, Roberts GD, Temesgen Z. Rhino-orbitocerebral mucormycosis caused by *Apophysomyces elegans*. J Clin Microbiol. 2006;44:892–8. DOI: 10.1128/JCM.44.3.892-898.2006
6. Bouza E, Munoz P, Guinea J. Mucormycosis: an emerging disease? Clin Microbiol Infect. 2006;12(Suppl. 7):7–23. DOI: 10.1111/j.1469-0691.2006.01604.x
7. Chayakulkeeree M, Ghannoum MA, Perfect JR. Zygomycosis: the re-emerging fungal infection. Eur J Clin Microbiol Infect Dis. 2006;25:215–29. DOI: 10.1007/s10096-006-0107-1
8. Roden MM, Zaoutis TE, Buchanan WL, Knudsen TA, Sarkisova TA, Schaufele RL, et al. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. Clin Infect Dis. 2005;41:634–53. DOI: 10.1086/432579
9. Chamilos G, Lewis RE, Kontoyiannis DP. Delaying amphotericin B–based frontline therapy significantly increases mortality among patients with hematologic malignancy who have zygomycosis. Clin Infect Dis. 2008;47:503–9. DOI: 10.1086/590004
10. Marty FM, Cosimi LA, Baden LR. Breakthrough zygomycosis after voriconazole treatment in recipients of hematopoietic stem-cell transplants. N Engl J Med. 2004;350:950–2. DOI: 10.1056/NEJM200402263500923
11. Dannaoui E, Meis JF, Loebenberg D, Verweij PE. Activity of posaconazole in treatment of experimental disseminated zygomycosis. Antimicrob Agents Chemother. 2003;47:3647–50. DOI: 10.1128/AAC.47.11.3647-3650.2003
12. Greenberg RN, Mullane K, van Burik JA, Raad I, Abzug MJ, Anstead G, et al. Posaconazole as salvage therapy for zygomycosis. Antimicrob Agents Chemother. 2006;50:126–33. DOI: 10.1128/AAC.50.1.126-133.2006
13. van Burik JA, Hare RS, Solomon HF, Corrado ML, Kontoyiannis DP. Posaconazole is effective as salvage therapy in zygomycosis: a retrospective summary of 91 cases. Clin Infect Dis. 2006;42:e61–5. DOI: 10.1086/500212
14. Soummer A, Mathonnet A, Scatton O, Massault PP, Paugam A, Lemiale V, et al. Failure of deferasirox, an iron chelator agent, combined with antifungals in a case of severe zygomycosis. Antimicrob Agents Chemother. 2008;52:1585–6. DOI: 10.1128/AAC.01611-07
15. Spellberg B, Andes D, Perez M, Anglim A, Bonilla H, Mathisen GE, et al. Safety and outcomes of open-label deferasirox iron chelation therapy for mucormycosis. Antimicrob Agents Chemother. 2009;53:3122–5.
16. Prabhu RM, Patel R. Mucormycosis and entomophthoramycosis: a review of the clinical manifestations, diagnosis and treatment. Clin Microbiol Infect. 2004;10(Suppl 1):31–47. DOI: 10.1111/j.1470-9465.2004.00843.x
17. Kontoyiannis DP, Lionakis MS, Lewis RE, Chamilos G, Healy M, Perego C, et al. Zygomycosis in a tertiary-care cancer center in the era of aspergillus-active antifungal therapy: a case–control observational study of 27 recent cases. J Infect Dis. 2005;191:1350–60. DOI: 10.1086/428780
18. Singh N, Aguadao JM, Bonatti H, Forrest G, Gupta KL, Safdar N, et al. Zygomycosis in solid organ transplant recipients: a prospective, matched case–control study to assess risks for disease and outcome. J Infect Dis. In press.
19. Siwek GT, Dodgson KJ, de Magalhaes-Silverman M, Bartelt LA, Kilborn SB, Hoth PL, et al. Invasive zygomycosis in hematopoietic stem cell transplant recipients receiving voriconazole prophylaxis. Clin Infect Dis. 2004;39:584–7. DOI: 10.1086/422723
20. Vigouroux S, Morin O, Moreau P, Mechinaud F, Morineau N, Mahe B, et al. Zygomycosis after prolonged use of voriconazole in immunocompromised patients with hematologic disease: attention required. Clin Infect Dis. 2005;40:e35–7. DOI: 10.1086/427752
21. Buisson G. PMSI- Redressements du programme de médicalisation des systèmes d'informations, N°80, March 2005. Séries Statistiques—DREES 2007 [cited 2008 May 21]. Available from http://www.sante.gouv.fr/drees/seriestat/seriestat80.htm
22. Gallay A, Vaillant V, Bouvet P, Grimont P, Desenclos JC. How many foodborne outbreaks of *Salmonella* infection occurred in France in 1995? Application of the capture–recapture method to three surveillance systems. Am J Epidemiol. 2000;152:171–7. DOI: 10.1093/aje/152.2.171

23. Rees JR, Pinner RW, Hajjeh RA, Brandt ME, Reingold AL. The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992–1993: results of population-based laboratory active surveillance. Clin Infect Dis. 1998;27:1138–47. DOI: 10.1086/514975
24. Torres-Narbona M, Guinea J, Martinez-Alarcon J, Munoz P, Gadea I, Bouza E. Impact of zygomycosis on microbiology workload: a survey study in Spain. J Clin Microbiol. 2007;45:2051–3. DOI: 10.1128/JCM.02473-06
25. Torres-Narbona M, Guinea J, Martinez-Alarcon J, Munoz P, Pelaez T, Bouza E. Workload and clinical significance of the isolation of zygomycetes in a tertiary general hospital. Med Mycol. 2008;46:225–30. DOI: 10.1080/13693780701796973
26. Trevejo RT. Acute encephalitis hospitalizations, California, 1990–1999: unrecognized arboviral encephalitis? Emerg Infect Dis. 2004;10:1442–9.
27. Chang DC, Burwell LA, Lyon GM, Pappas PG, Chiller TM, Wannemuehler KA, et al. Comparison of the use of administrative data and an active system for surveillance of invasive aspergillosis. Infect Control Hosp Epidemiol. 2008;29:25–30. DOI: 10.1086/524324
28. Carré N, Uhry Z, Velten M, Tretarre B, Schvartz C, Molinie F, et al. Predictive value and sensibility of hospital discharge system (PMSI) compared to cancer registries for thyroid cancer (1999–2000) [in French]. Rev Epidemiol Sante Publique. 2006;54:367–76. DOI: 10.1016/S0398-7620(06)76731-1
29. Geoffroy-Perez B, Imbernon E, Gilg S, I, Goldberg M. Comparison of the French DRG based information system (PMSI) with the National Mesothelioma Surveillance Program database.[In French]. Rev Epidemiol Sante Publique. 2006;54:475–83. DOI: 10.1016/S0398-7620(06)76747-5
30. Hafdi-Nejjari Z, Couris CM, Schott AM, Perrot L, Bourgoin F, Borson-Chazot F, et al. Role of hospital claims databases from care units for estimating thyroid cancer incidence in the Rhone-Alpes region of France [in French]. Rev Epidemiol Sante Publique. 2006;54:391–8. DOI: 10.1016/S0398-7620(06)76737-2
31. Tricco AC, Pham B, Rawson NS. Manitoba and Saskatchewan administrative health care utilization databases are used differently to answer epidemiologic research questions. J Clin Epidemiol. 2008;61:192–7. DOI: 10.1016/j.jclinepi.2007.03.009
32. Marr KA, Carter RA, Crippa F, Wald A, Corey L. Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin Infect Dis. 2002;34:909–17. DOI: 10.1086/339202
33. Blin N, Morineau N, Gaillard F, Morin O, Milpied N, Harousseau JL, et al. Disseminated mucormycosis associated with invasive pulmonary aspergillosis in a patient treated for post-transplant high-grade non-Hodgkin's lymphoma. Leuk Lymphoma. 2004;45:2161–3. DOI: 10.1080/10428190410001700803
34. Girmenia C, Moleti ML, Micozzi A, Iori AP, Barberi W, Foa R, et al. Breakthrough *Candida krusei* fungemia during fluconazole prophylaxis followed by breakthrough zygomycosis during caspofungin therapy in a patient with severe aplastic anemia who underwent stem cell transplantation. J Clin Microbiol. 2005;43:5395–6. DOI: 10.1128/JCM.43.10.5395-5396.2005
35. Imhof A, Balajee SA, Fredricks DN, Englund JA, Marr KA. Breakthrough fungal infections in stem cell transplant recipients receiving voriconazole. Clin Infect Dis. 2004;39:743–6. DOI: 10.1086/423274
36. Agence de la Biomedecine. Rapport d'activité de l'Agence de la Biomedecine 2007, 234 p. [cited 2009 May 20]. Available from http://www.agence-biomedecine.fr/uploads/document/rapp-synth2007.pdf
37. Kusnik-Joinville O, Weill A, Salanave B, Ricordeau P, Allemand H. Prevalence and treatment of diabetes in France: trends between 2000 and 2005. Diabetes Metab. 2008;34:266–72. DOI: 10.1016/j.diabet.2008.01.005
38. Pagano L, Caira M, Candoni A, Offidani M, Fianchi L, Martino B, et al. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica. 2006;91:1068–75.
39. Chakrabarti A, Das A, Mandal J, Shivaprakash MR, George VK, Tarai B, et al. The rising trend of invasive zygomycosis in patients with uncontrolled diabetes mellitus. Med Mycol. 2006;44:335–42. DOI: 10.1080/13693780500464930
40. Perlroth J, Choi B, Spellberg B. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med Mycol. 2007;45:321–46. DOI: 10.1080/13693780701218689

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Chicken Consumption and Use of Acid-Suppressing Medications as Risk Factors for *Campylobacter* Enteritis, England

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In a case–control study of *Campylobacter* spp. risk factors in England during 2005–2006, we identified recent consumption of commercially prepared chicken as an important risk factor. The risk for illness associated with recent chicken consumption was much lower for persons who regularly ate chicken than in those who did not, suggesting that partial immunologic protection may follow regular chicken preparation or consumption. Chicken-related risk factors accounted for 41% of cases; acid-suppressing medication, for 10%; self-reported past *Campylobacter* enteritis, 2%; and recent acquisition of a pet dog, 1%. Understanding the risks associated with chicken from different sources will benefit strategies to reduce *Campylobacter* infections. Better characterization of immune correlates for *Campylobacter* infection is necessary to assess the relative importance of immunity and behavioral factors in determining risk.

Campylobacter spp. are the most common bacterial cause of enteritis in England. More than 40,000 cases are reported annually (*1*). Incidence of cases reported nationally is ≈80 per 100,000 population, but the community incidence is ≈7× higher (*2*). Previously identified risk factors for *Campylobacter* enteritis include international travel; ingestion of poultry, red meat, unpasteurized milk, and untreated water; contact with pets and farm animals; use of antimicrobial drugs and acid-suppressing medication; and diabetes (*3–11*).

Numerous studies implicate chicken consumption as an important risk factor for *Campylobacter* enteritis (*6–18*). However, some studies report associations specifically with eating undercooked chicken (*5*,*6*,*12*); others, with any type of chicken; and in 1 study, chicken consumption appeared to be protective (*19*). Other studies have found increased risks only with consumption of commercially prepared chicken (*6–8*,*11*,*14*,*20*).

One explanation for these disparities is that studies generally measure the average increase in risk from chicken consumption, without accounting for differences in individual susceptibility. We hypothesized that the frequency of chicken consumption modifies risk for *Campylobacter* enteritis associated with recent chicken consumption, possibly because persons who regularly eat chicken develop partial immunity to *Campylobacter* infection or because they have different consumption or preparation behaviors that influence risk for infection. We report the results of a multicenter case-control study in England designed to investigate food and other risk factors for reported *Campylobacter* enteritis.

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DOI: 10.3201/eid1509.080773

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Methods

Study Participants

Cases were laboratory-confirmed *Campylobacter* spp. infections in persons ≥18 years of age reported to 1 of 5 English Health Protection Units (HPUs) (East Midlands North, Cheshire and Merseyside, Cumbria and Lancashire, North East and Central London, and Essex) from April 1, 2005, through June 30, 2006. We randomly selected 5 controls per case from records of all persons registered with primary care clinics in the area. Controls were stratum-matched to cases by HPU, age group (18–34, 35–54, and ≥55 years), sex, and month of report.

Exclusion criteria were international travel in the 14 days before illness for case-patients (or questionnaire completion for controls) and preexisting irritable bowel syndrome. Household clusters were identified by surname and postal address; only the first case in household clusters was included. Controls reporting gastrointestinal symptoms in the preceding 14 days also were excluded.

Case and Control Recruitment

We recruited case-patients by mail through their local Environmental Health Department or HPU and asked them to return a postage-paid self-completed risk factor questionnaire. We recruited controls by mail through the Health Protection Agency Centre for Infections and asked them to complete a similar questionnaire. Reminders were sent to nonresponders after 2 and 3 weeks. Signed, informed consent was obtained from participants.

Data Collection

We inquired about demographic information, clinical details, and risk factors in the 5 days before illness for cases and questionnaire completion for controls (i.e., 5-day factors). We also collected information about routine exposures (i.e., habitual factors).

Statistical Analysis

Risk factors were grouped under 7 domains: health, occupation, pets, water, recreational exposures, food, and household details. We analyzed data by unconditional logistic regression by using Stata 8.2 software (Stata Corporation, College Station, TX, USA). ORs and 95% confidence intervals (CIs) were calculated for each exposure. Analyses were adjusted for age group (18–24, 25–34, 35–44, 45–54, 55–64, and ≥65 years), sex, study site, and calendar month.

We powered our study to detect an odds ratio (OR) of 1.4 for chicken consumption in the previous week (87% population prevalence (*21*), power = 0.8, $\alpha = 0.05$), or an OR of 2.4 for an exposure with 1% prevalence.

Final Multivariable Model

Within each exposure domain, we first constructed a model comprising all habitual exposures. This model was simplified by using backward stepwise elimination until all remaining variables yielded likelihood ratio (LR) test results of $p<0.05$. This process was repeated for 5-day exposure variables and conducted separately for each exposure domain.

Next, we fitted a model comprising all habitual variables identified in the domain-specific regressions and simplified by backward stepwise elimination as before. A model for all 5-day exposures identified in the domain-specific regressions was similarly constructed.

Lastly, all habitual and 5-day factors from the above regressions were included in 1 model and the final model obtained by backward stepwise elimination. For all risk factors positively associated with disease, we also calculated the proportion of cases attributable to each risk factor (population-attributable fraction).

Chicken Consumption (Interaction Model)

We investigated further whether regular consumption of chicken modified the risk for disease from recent chicken consumption. We classified participants according to whether they 1) regularly ate chicken (at least once a week) and 2) had eaten it in the previous 5 days. We further classified persons who had eaten chicken in the previous 5 days according to whether they ate it in their own or someone else's home, at a commercial establishment, or both. We fit a model with an interaction between these variables to investigate how the risk for disease varied in these subgroups relative to persons not exposed to chicken (defined as reporting they did not regularly eat chicken and had not eaten it in the previous 5 days). We assessed statistical evidence for the interaction using the LR test. In a separate model, we additionally adjusted for all other risk factors identified in the multivariable analysis. Because of small numbers in some subgroups, this latter analysis could be performed only for persons who regularly ate chicken and reported eating it in the previous 5 days.

Sensitivity Analysis

For each of the final multivariable and interaction models, we conducted 2 sensitivity analyses. First, we repeated the analysis excluding case-patients for whom the delay between symptoms onset and questionnaire completion was longer than the median delay for all case-patients. We compared the ORs from this model to those from the model comprising all case-patients to explore potential effects of differential reporting of risk factors among late responders. In the interaction model, we could perform this analysis only for persons who regularly ate chicken and reported

eating it in the previous 5 days because of small numbers in other subgroups.

Second, by using an inverse probability-weighted approach (*22*), we investigated whether differences between participants and nonparticipants influenced results. We calculated individuals' probabilities of participation from a 2-level random intercept logistic model regressing study participation against study site; a 3-way interaction between case and control status, age group, and sex; and area-level deprivation. To account for differences in area-level deprivation, we linked individuals' postcodes to super output areas (SOAs), geographic boundaries comprising ≈1,000 residents for which aggregated census data are available. SOAs are ranked according to the Index of Multiple Deprivation (*23*), which scores SOAs on 7 domains related to unemployment, income, education, housing, living environment, crime, and healthcare access. We modeled area-level deprivation using SOA as a latent, random intercept variable at the higher level. We then used the inverse probabilities of participation from this model as weights in the final multivariable and interaction models, effectively giving more weight to persons in strata with low participation. We compared the ORs from the weighted and unweighted models to assess potential participation bias.

Ethical Approval

This study received a favorable ethical opinion from the North West Multicentre Research Ethics Committee. Approval was obtained from local research management and governance departments serving each study site.

Results

A total of 2,381 (46.5%) case-patients and 5,256 (37.3%) controls returned questionnaires. Participants were excluded for the following reasons: missing age information (2 case-patients, 7 controls); chronic gastrointestinal illness (221 case-patients, 324 controls); gastrointestinal symptoms in the preceding 14 days (431); international travel in the preceding 14 days (560 case-patients, 511 controls); and being part of a household cluster of gastrointestinal illness (6 case-patients). After exclusions, 1,592 cases and 3,983 controls were available for analysis. Among controls, 2,486 (62.4%), 700 (17.6%), and 689 (17.3%) questionnaires were completed after the initial contact, first reminder, and second reminder respectively. Date of questionnaire completion was unknown or implausible for 108 controls.

Single-Variable Analysis

Habitual factors associated with increased risk were self-reported diarrheal illness in the previous 12 months; self-reported past *Campylobacter* enteritis; use of antimicrobial drugs, antacids and acid-suppressing medications in the previous 28 days; diabetes; puppy ownership; recent dog acquisition; chicken consumption at least once a week; red meat consumption once a week; and sharing of kitchen facilities. Eating commercially prepared chicken in the previous 5 days also was associated with increased risk (Table 1).

Habitual factors associated with decreased risk for illness were vegetarianism; regular consumption of salads, rice, and legumes; occupational exposure to sheep and horses; ownership of fish or rodents; and regular drinking of unpasteurized milk. Consumption of unpasteurized milk and dairy products, noncarbonated and carbonated bottled water, and unfiltered tap water in the previous 5 days also was associated with decreased risk.

Final Multivariable Model

In the final model, positively associated exposures were past *Campylobacter* enteritis (OR 2.2, 95% CI 1.3–3.6), recent use of acid-suppressing medication (OR 3.4, 95% CI 2.5–4.6), recent acquisition of a dog (OR 14.4, 95% CI 3.7–54.1), regular consumption of chicken (OR 3.7, 95% CI 2.1–6.8 for those eating chicken ≥5 times a week), and consumption of commercially prepared chicken only in the previous 5 days (OR 2.0, 95% CI 1.3–3.0). Regular consumption of salads, legumes, and unpasteurized milk and consumption of home-prepared chicken in the previous 5 days were associated with decreased risk (Table 2).

Chicken Consumption (Interaction Model)

Statistical evidence was strong for an interaction between regular and recent chicken consumption (LR test p = 0.0002) (Figure). Overall, persons who regularly ate chicken (at least once a week) were at greater risk for illness than those who did not (OR 1.6, 95% CI 1.2–2.0) (Figure, top). However, for persons who did not regularly eat chicken, eating it in the previous 5 days posed a 5-fold greater risk than it did for persons who did not (OR 5.0, 95% CI 2.1–11.9). We did not see this association for persons who regularly ate chicken (OR 0.8, 95% CI 0.6–1.0) (Figure, middle).

The risk associated with eating commercially prepared chicken was greater than that associated with eating home-prepared chicken. Among persons who regularly ate chicken, eating commercially prepared chicken in the previous 5 days was associated with a 4-fold increased risk (OR 4.0, 95% CI 2.8–5.8) for *Campylobacter* infection, much higher than the risk associated with eating home-prepared chicken (OR 1.5, 95% CI 1.1–2.1). Among those who did not regularly eat chicken, eating commercially prepared chicken was associated with a 36-fold increased risk (OR 35.7, 95% CI 3.7–344.1); however, this group was very small (Figure, bottom).

Adjusting for nonchicken-related factors had little effect on the ORs (Table 2). The p values were considerably higher, although this analysis was based on fewer persons because of missing data in some variables.

Sensitivity Analyses

Excluding late-responding case-patients had little effect on the ORs in either the final multivariable model or the interaction model. In the final multivariable model, ORs for eating chicken ≥1 times per week were consistently higher than in the model comprising all cases (OR 5.4, 95% CI 2.3–12.4 for eating chicken ≥5 times per week).

In the inverse probability-weighted final model, the OR for eating commercially prepared chicken in the previous 5 days was 1.6 (95% CI 0.98–2.62). Other results did

Table 1. Final multivariable model of both habitual risk factors and risk factors for *Campylobacter* enteritis in the previous 5 days, adjusted for participant age group and sex, study site, and month of year, England, 2005–2006

Exposure domain and variable	Odds ratio	95% Confidence interval	p value
Health details			
Previous *Campylobacter* infection	2.20	1.33–3.64	0.002
Use of acid-suppressing medication in previous 28 days	3.39	2.49–4.62	<0.001
Pets			
Pet fish	0.56	0.33–0.94	0.029
If last pet acquired was a dog, how long ago was it acquired?			
Dog was not last pet bought/no pets	1.00	–	–
≥6 months ago	0.76	0.57–1.01	0.057
3–6 months ago	1.30	0.53–3.16	0.566
1–3 months ago	1.74	0.62–4.93	0.296
2–4 weeks ago	14.40	3.69–56.14	<0.001
<2 weeks ago	1.08	0.12–9.90	0.946
Food			
No. times salads eaten per week			
0	1.00	–	–
1	0.89	0.63–1.26	0.503
2	0.58	0.40–0.82	0.002
3	0.72	0.49–1.05	0.086
4	0.93	0.62–1.40	0.739
≥5	0.63	0.44–0.91	0.013
No. times legumes eaten per week			
0	1.00	–	–
1	0.65	0.51–0.84	0.001
2	0.57	0.44–0.75	<0.001
3	0.47	0.33–0.68	<0.001
4	0.65	0.40–1.05	0.078
≥5	0.66	0.42–1.04	0.071
No. times fruit eaten per week			
0	1.00	–	–
1	0.95	0.53–1.69	0.860
2	1.57	0.96–2.55	0.071
3	1.19	0.71–1.98	0.518
4	1.77	1.05–2.98	0.032
≥5	1.06	0.70–1.61	0.775
No. times chicken eaten per week			
0	1.00	–	–
1	1.62	0.98–2.68	0.058
2	1.96	1.16–3.32	0.012
3	1.70	0.98–2.95	0.061
4	2.10	1.16–3.79	0.014
≥5	3.74	2.06–6.80	<0.001
Regularly drinks raw milk	1.00	–	–
Rarely/never			
Yes, regularly	0.24	0.08–0.72	0.010
Yes, occasionally	0.70	0.33–1.51	0.365
Location where chicken eaten in past 5 days was prepared			
No chicken eaten	1.00	–	–
In the home/someone else's home only	0.70	0.49–1.00	0.050
Outside the home only	1.95	1.26–3.01	0.003
In the home and outside the home	0.70	0.48–1.03	0.069

Table 2. Comparison of increased risks for *Campylobacter* enteritis associated with eating chicken in the previous 5 days in persons who regularly ate and never ate chicken, England, 2005–2006*

Regularly eats chicken	Ate chicken in previous 5 days	Location where chicken was prepared	OR†	95% CI	p value
No	No	–	1.00	–	–
Yes	Yes	In the home only	1.47	0.96–2.26	0.078
Yes	Yes	Outside the home only	3.86	2.33–6.39	<0.001
Yes	Yes	Inside the home, and prepared outside the home	1.59	1.02–2.47	0.042

*OR, odds ratio; CI, confidence interval.
†ORs adjusted for participant age group and sex; study site; study month; use of acid suppressing medication; self-reported past *Campylobacter* enteritis; recent acquisition of a dog; and frequency of consuming of salads, fruit, vegetables, and unpasteurized milk.

not change. In the interaction model, the weighted model indicated stronger evidence for associations with eating home-prepared chicken (OR 1.76, 95% CI 1.22–2.29, p = 0.001) and eating home-prepared and commercially prepared chicken in the previous 5 days (OR 1.63, 95% CI 1.18–2.25, p = 0.003), compared with the unweighted results (Table 1).

Population-Attributable Fractions

Chicken-related exposures were reported by 92.5% of controls; use of acid-suppressing medications, by 6.0%; past *Campylobacter* enteritis, by 2.2%; and recent acquisition of a dog, by 1.6%. The percentage of cases attributable to each of these risk factors (Table 3) was as follows: chicken-related exposures, 41%; acid-suppressing medications, 10%; past *Campylobacter* enteritis, 3%; and recent acquisition of a dog, 1%.

Discussion

Chicken consumption and use of acid-suppressing medications are major risk factors for *Campylobacter* enteritis in England. Chicken-related exposures accounted for 41% of adult cases, consistent with previous US and Australian studies (*5*,*8*,*24*). Recent use of acid-suppressing medications increased risk for illness 3-fold, similar to other studies (*9*), accounting for 10% of cases.

Like others (*3*,*6*–*8*,*11*), we found that commercially prepared chicken poses a greater risk than home-prepared chicken. Reasons might be greater contamination levels or inadequate cooking procedures, which could be more common in commercial establishments than in homes. However, we found only modest increases in risk for persons who ate home-prepared and commercially prepared chicken, suggesting that persons who regularly eat chicken at home frequent different types of establishments than do persons who tend to eat chicken only outside the home. We could not investigate this hypothesis further.

Several findings suggest that acquired immunity might be important. The risk for *Campylobacter* enteritis associated with recent chicken consumption depended on whether participants regularly ate chicken. For persons who ate chicken in the previous 5 days, the risk was considerably greater for those who did not regularly eat chicken than for those who did. Recent, but not longer-term, dog owners had higher risk for illness, whereas persons who regularly drank unpasteurized milk had decreased risk. We could not confirm participants' immunologic status; however, these results suggest that long-term exposure to these sources of *Campylobacter* spp. might confer partial immunity (*25*). In immunologically susceptible populations, however, unpasteurized milk is a well-known cause of outbreaks of infection with *Campylobacter* and potentially fatal Shiga–toxin producing *Escherichia coli* (*26*). Further developments to characterize relevant correlates of immune status for *Campylobacter* infection are required to confirm these findings.

Despite the potential role of immunity, participants reporting previous *Campylobacter* enteritis, but not nonspecific enteritis, had greater risk for recent *Campylobacter* illness

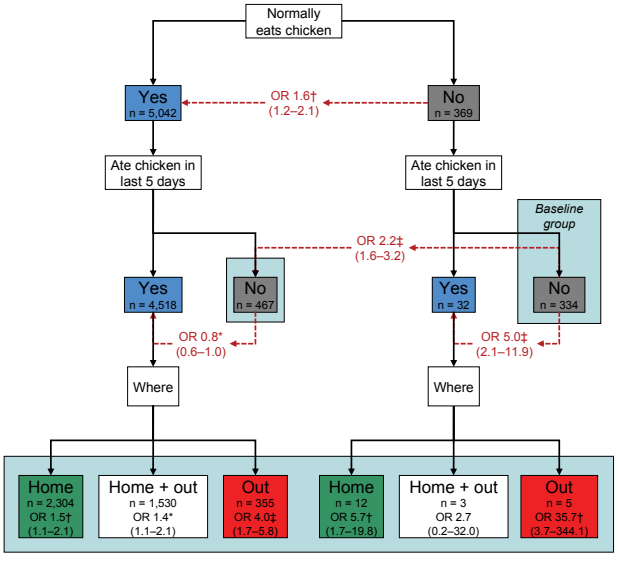

Figure. Odds ratios (ORs) and 95% confidence intervals (CIs) for *Campylobacter* enteritis associated with chicken consumption, England, 2005–2006. Numbers in boxes represent persons in each category; Numbers in red are ORs and 95% CIs for relevant comparisons; arrows indicate direction of risk. For boxes in the bottom level, ORs compare risk for *Campylobacter* enteritis between individuals in that group and the baseline group (labeled), which comprises persons who do not regularly eat chicken and did not eat chicken in the previous 5 days (n = 334). Model is adjusted for age group, sex, study site, and month. *p<0.05; †p<0.01; ‡p<0.001.

Table 3. Population–attributable fractions for identified risk factors for *Campylobacter* enteritis, England, 2005–2006*

Variable	PAF, %	SE	95% CI
Previous *Campylobacter* infection	2.6	1.37	0.0–5.3
Proton pump inhibitor use in previous 28 days	10.4	1.70	7.0–13.6
Acquisition of dog in previous month	1.2	1.10	0.0–3.3
Chicken consumption	40.6	11.84	12.2–59.8

*PAF, population–attributable fraction; CI, confidence interval.

than did persons not reporting past *Campylobacter* enteritis. Compared with all cases, those reporting a previous episode of *Campylobacter* enteritis were of similar age but more likely to be female (57% vs. 49%). These persons may differ in other ways that increase risk, such as medical history or immune competence. However, this finding should be interpreted cautiously because we had no independent confirmation of self-reported *Campylobacter* enteritis.

Other researchers (*11*) have suggested that regular consumption of vegetables and legumes might protect against infection. However, eating these foods might simply be a marker for unmeasured behavior related to decreased risk.

We found no associations with any environmental variables. Environmental exposures may pose low or transient risk; temporal variation in environmental prevalence of *Campylobacter* spp. could make their effects difficult to detect. Previous studies in England have identified diabetes as a risk factor for *Campylobacter* enteritis (*9*); in our study, initial analyses suggested a 1.5-fold increase in illness associated with diabetes, but this effect disappeared after adjustment for other habitual factors.

We did not include persons who reported recent international travel because travel-related illness may have different risk factors. However, international travel is common among persons in England with laboratory-confirmed *Campylobacter* infection; 24% of all case-patients reported traveling abroad in the previous 14 days compared with 11% of controls.

Our analysis emphasizes the importance of accounting for regular dietary habits in determining risk associated with recent consumption of putatively risky foods. Moreover, selection of an appropriate baseline comparison group (in this case, persons truly unexposed to chicken consumption) is crucial to enable meaningful comparisons. In the future, distinguishing long-term and recent exposures will be important in investigating how their association influences risk. More detailed study of the risks associated with chicken prepared at home and in commercial establishments is needed. Given the limitations of case–control studies for collecting long-term exposure information, innovative studies using a variety of approaches are necessary.

In England, chicken consumption is the major recognized risk factor for *Campylobacter* enteritis. Understanding the differing risks from poultry sources should guide strategies to reduce risk for infection from chicken. Immunologic factors appear to be important in determining risk for *Campylobacter* enteritis given exposure to infection. Meaningful interpretation of *Campylobacter* risk factor studies requires better knowledge of population susceptibility to infection and the extent to which past exposure can induce protection. Identifying relevant immune correlates would help determine whether differences in immune status, behavior, or both are responsible for differing risks for *Campylobacter* enteritis between populations or population subgroups.

Acknowledgments

We thank Tracey Wood and Maria Davies for assistance with data collection in Cheshire and Merseyside and staff at the Environmental Health Departments at the respective study sites for supplying data for the study.

This study was funded by the United Kingdom Food Standards Agency (project no. B14011).

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References

1. Health Protection Agency. *Campylobacter* spp.: laboratory reports of faecal isolates reported to the Health Protection Agency Centre for Infections [cited 2008 Feb 1]. Available from http://www.hpa.org.uk/infections/topics_az/campy/data_ew.htm
2. Wheeler JG, Sethi D, Cowden JM, Wall PG, Rodrigues LC, Tompkins DS, et al. Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. The Infectious Intestinal Disease Study Executive. BMJ. 1999;318:1046–50.
3. Unicomb LE, Dalton CB, Gilbert GL, Becker NG, Patel MS. Age-specific risk factors for sporadic *Campylobacter* infection in regional Australia. Foodborne Pathog Dis. 2008;5:79–85. DOI: 10.1089/fpd.2007.0047
4. Gallay A, Bousquet V, Siret V, Prouzet-Mauleon V, Valk H, Vaillant V, et al. Risk factors for acquiring sporadic *Campylobacter* infection in France: results from a national case–control study. J Infect Dis. 2008;197:1477–84. DOI: 10.1086/587644
5. Stafford RJ, Schluter P, Kirk M, Wilson A, Unicomb L, Ashbolt R, et al. A multi-centre prospective case–control study of *Campylobacter* infection in persons aged 5 years and older in Australia. Epidemiol Infect. 2007;135:978–88. DOI: 10.1017/S0950268806007576
6. Eberhart-Phillips J, Walker N, Garrett N, Bell D, Sinclair D, Rainger W, et al. Campylobacteriosis in New Zealand: results of a case–control study. J Epidemiol Community Health. 1997;51:686–91. DOI: 10.1136/jech.51.6.686

7. Effler P, Ieong MC, Kimura A, Nakata M, Burr R, Cremer E, et al. Sporadic *Campylobacter jejuni* infections in Hawaii: associations with prior antibiotic use and commercially prepared chicken. J Infect Dis. 2001;183:1152–5. DOI: 10.1086/319292
8. Friedman CR, Hoekstra RM, Samuel M, Marcus R, Bender J, Shiferaw B, et al. Risk factors for sporadic *Campylobacter* infection in the United States: a case–control study in FoodNet sites. Clin Infect Dis. 2004;38(Suppl 3):S285–96. DOI: 10.1086/381598
9. Neal KR, Slack RC. Diabetes mellitus, anti-secretory drugs and other risk factors for *Campylobacter* gastro-enteritis in adults: a case–control study. Epidemiol Infect. 1997;119:307–11. DOI: 10.1017/S0950268897008224
10. Neimann J, Engberg J, Molbak K, Wegener HC. A case–control study of risk factors for sporadic *Campylobacter* infections in Denmark. Epidemiol Infect. 2003;130:353–66.
11. Rodrigues LC, Cowden JM, Wheeler JG, Sethi D, Wall PG, Cumberland P, et al. The study of infectious intestinal disease in England: risk factors for cases of infectious intestinal disease with *Campylobacter jejuni* infection. Epidemiol Infect. 2001;127:185–93. DOI: 10.1017/S0950268801006057
12. Ikram R, Chambers S, Mitchell P, Brieseman MA, Ikam OH. A case–control study to determine risk factors for *Campylobacter* infection in Christchurch in the summer of 1992–3. N Z Med J. 1994;107:430–2.
13. Kapperud G, Skjerve E, Bean NH, Ostroff SM, Lassen J. Risk factors for sporadic *Campylobacter* infections: results of a case–control study in southeastern Norway. J Clin Microbiol. 1992;30:3117–21.
14. Michaud S, Menard S, Arbeit RD. Campylobacteriosis, Eastern Townships, Quebec. Emerg Infect Dis. 2004;10:1844–7.
15. Studahl A, Andersson Y. Risk factors for indigenous *Campylobacter* infection: a Swedish case–control study. Epidemiol Infect. 2000;125:269–75. DOI: 10.1017/S0950268899004562
16. Tenkate TD, Stafford RJ. Risk factors for *Campylobacter* infection in infants and young children: a matched case–control study. Epidemiol Infect. 2001;127:399–404. DOI: 10.1017/S0950268801006306
17. Wingstrand A, Neimann J, Engberg J, Nielsen EM, Gerner-Smidt P, Wegener HC, et al. Fresh chicken as main risk factor for campylobacteriosis, Denmark. Emerg Infect Dis. 2006;12:280–5.
18. Danis K, Di Renzi M, O'Neill W, Smyth B, McKeown P, Foley B, et al. Risk factors for sporadic *Campylobacter* infection: an all-Ireland case–control study. Euro Surveill. 2009;14:pii=19123.
19. Adak GK, Cowden JM, Nicholas S, Evans HS. The Public Health Laboratory Service national case–control study of primary indigenous sporadic cases of *Campylobacter* infection. Epidemiol Infect. 1995;115:15–22. DOI: 10.1017/S0950268800058076
20. Evans MR, Ribeiro CD, Salmon RL. Hazards of healthy living: bottled water and salad vegetables as risk factors for *Campylobacter* infection. Emerg Infect Dis. 2003;9:1219–25.
21. Infectious Intestinal Disease Study Team. A report of the study of infectious intestinal disease in England. London: The Stationery Office; 2000.
22. Cole SR, Hernan MA, Robins JM, Anastos K, Chmiel J, Detels R, et al. Effect of highly active antiretroviral therapy on time to acquired immunodeficiency syndrome or death using marginal structural models. Am J Epidemiol. 2003;158:687–94. DOI: 10.1093/aje/kwg206
23. The English indices of deprivation 2004: summary (revised) [cited 2009 Jun 30]. Available from http://www.rbkc.gov.uk/kcpartnership/General/pc_indices_deprivation.pdf
24. Stafford RJ, Schluter PJ, Wilson AJ, Kirk MD, Hall G, Unicomb L. Population-attributable risk estimates for risk factors associated with *Campylobacter* infection, Australia. Emerg Infect Dis. 2008;14:895–901. DOI: 10.3201/eid1406.071008
25. Blaser MJ, Sazie E, Williams LP Jr. The influence of immunity on raw milk–associated *Campylobacte*r infection. JAMA. 1987;257:43–6. DOI: 10.1001/jama.257.1.43
26. Frost JA, Gillespie IA, O'Brien SJ. Public health implications of *Campylobacter* outbreaks in England and Wales, 1995–9: epidemiological and microbiological investigations. Epidemiol Infect. 2002;128:111–8. DOI: 10.1017/S0950268802006799

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Genetic Characterization of Foot-and-Mouth Disease Viruses, Ethiopia, 1981–2007

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Foot-and-mouth disease (FMD) is endemic to sub-Saharan Africa. To further understand its complex epidemiology, which involves multiple virus serotypes and host species, we characterized the viruses recovered from FMD outbreaks in Ethiopia during 1981–2007. We detected 5 of the 7 FMDV serotypes (O, A, C, Southern African Territories [SAT] 1, and SAT 2). Serotype O predominated, followed by serotype A; type C was not recognized after 1983. Phylogenetic analysis of virus protein 1 sequences indicated emergence of a new topotype within serotype O, East Africa 4. In 2007, serotype SAT 1 was detected in Ethiopia and formed a new distinct topotype (IX), and serotype SAT 2 reappeared after an apparent gap of 16 years. The diversity of viruses highlights the role of this region as a reservoir for FMD virus, and their continuing emergence in Ethiopia will greatly affect spread and consequent control strategy of the disease on this continent.

Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious and economically devastating disease of cloven-hooved domestic and wild animals. It can cause a high number of deaths among young animals and production losses in adults and is the single most limiting constraint to international trade of live animals and animal products. FMDV belongs to the genus *Aphthovirus* in the family *Picornaviridae* and possesses a single strand of positive-sense RNA genome. It has a high mutation rate because the viral RNA–dependent RNA polymerase lacks proofreading ability, resulting in 7 immunogenically distinct serotypes (O, A, C, Southern African Territories [SAT] 1, SAT 2, SAT 3, and Asia 1) and numerous and constantly evolving variants showing a spectrum of antigenic diversity. Vaccination is an effective way to control FMD; however, the protection conferred by vaccination or infection is usually serotype specific and sometimes incomplete within a serotype (*1*–*3*).

FMD is endemic to sub-Saharan Africa; widespread outbreaks of clinical disease occur during most years (*4*,*5*). Of the 7 serotypes (except Asia 1), 6 have reportedly occurred on the continent, and disease control becomes more complicated because of marked regional differences in the distribution and prevalence of various serotypes and intratypic variants (*4*–*6*). FMD was first recorded in Ethiopia in 1957 when serotypes O and C were detected (*7*,*8*). Serotypes A and SAT 2 were not identified until 1969 and 1989, respectively (*8*,*9*). During 1988–1991, analysis of outbreak samples from Ethiopia at the National Veterinary Institute (NVI), Debre Zeit, Ethiopia, and at the Food and Agriculture Organization World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD), Institute for Animal Health, Pirbright, UK, identified serotype O and serotype SAT 2 FMDV as the causative agents (*9*). The occurrence of FMD in Ethiopia has apparently increased since 1990; outbreaks throughout the country are reported frequently (*10*). FMD remains largely uncontrolled in the country because vaccination for prophylactic purpose is not being practiced except for a few dairy herds containing exotic animals. With no control and preventive measures in place, FMD causes substantial economic loss to farmers and to the nation from embargoes of livestock and livestock product trade (*11*). To initiate control measures for FMD, the following must be identified: origin of infection, links between outbreaks, extent of genetic variation of the caus-

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DOI: 10.3201/eid1509.090091

ative viruses, and antigenic relationship of field isolates to the available vaccines.

Phylogenetic analysis of the virus protein (VP) 1 region of FMD viruses has been used extensively to investigate the molecular epidemiology of the disease worldwide. These techniques have helped define genetic relationships between FMDV isolates and geographic distribution of lineages and genotypes; they have also helped establish genetically and geographically linked topotypes and trace the source of outbreaks (*4,6,12*). Topotypes are defined as geographically clustered viruses that form a single genetic lineage generally sharing >85% (O, A, C, and Asia 1) or >80% (SAT 1, SAT 2, and SAT 3) nucleotide identity in the VP1-coding region.

We report the circulation of 4 of 7 serotypes of FMDV in Ethiopia: serotype O, serotype A, serotype SAT 2, and serotype SAT 1. Emergence of these viruses in Ethiopia will greatly affect spread and consequent control strategy of the disease on this continent because restriction of animal movement between many African countries is limited.

Materials and Methods

During 1981–2007, epithelial tissues and vesicular fluids were collected from FMD-suspected animals from different areas of Ethiopia and submitted to NVI in Debre Zeit. Bovine samples were collected throughout the country; swine samples were collected only from 1 swine farm (Alagae) in Zeway, Eastern Shoa, during 1986 and 1998; and ovine and caprine samples were collected from Mizan Teferi, Bench Maji, in 2007. No samples came from eastern Ethiopia. The samples were transported from the collection site to the diagnostic laboratory in 0.04 M phosphate buffer (pH 7.2–7.6) with 50% glycerol at 4°C (*13*) and stored at –20°C until tested (*14*). When possible, the same samples, or others collected at the same time as those tested at NVI, were also submitted to the WRLFMD in Pirbright for additional studies.

Laboratory Diagnosis

Viruses were isolated and serotypes were identified as follows. Established cell layers of either IB-RS-2 (porcine kidney) or BHK-21 (baby hamster kidney) at NVI or primary BTy (bovine thyroid) cells at WRLFMD were inoculated with the suspension of suspected material. Cytopathic effects were noted after 24–48 hours in positive samples. If no cytopathic effect was detected, the cells were passaged at least 1× more before the samples were declared negative. Serotyping of FMDV was carried out by complement fixation test at NVI (*13*) and by antigen-detection ELISA at WRLFMD (*15*).

Viruses and Primers

A selection of 81 viruses submitted to WRLFMD was further characterized by sequencing of the VP1 gene. The designation and origin of FMDV isolates studied are listed in online Technical Appendix Table 1 (available from www.cdc.gov/EID/content/15/9/1409-Techapp.pdf). Three alternative primer combinations were used for the reverse transcription–PCR (RT-PCR) of FMDV serotype O viruses: O-1C244F/EUR-2B52R, O-1C272F/EUR-2B52R, and O-1C283F/EUR-2B52R. Two primer sets were used for each of the other serotypes: serotype A (A-1C562F/EUR-2B52R and A-1C612F/EUR-2B52R), serotype C (C-1C536F/EUR-2B52R and C-1C616F/EUR-2B52R), serotype SAT 1 (SAT1-1C559F/SAT-2B208R and SAT-1U-OS/SAT-2B208R), and serotype SAT 2 (SAT2-P1-1223F/SAT 2B208R and SAT2-1C445F/SAT-2B208R) (online Technical Appendix Table 2). Additional internal sequencing primers were used to ensure coverage of the VP1 region on both strands (online Technical Appendix Table 2).

RT-PCR of Virus RNA

RNA extraction and RT-PCR were conducted according to the protocol described previously (*16*), except for the following. The thermal profiles used for amplification of the VP1 sequence of various serotypes were as follows: FMDV O: 42°C for 30 min, 94°C for 5 min, 35 cycles of 94°C for 60 s, 60°C for 60 s, and 72°C for 90 s, followed by a final extension of 72°C for 5 min. Conditions were the same for the other serotypes, except that extension temperatures were 55°C for A and C and 50°C for SAT 1 and SAT 2. After PCR, deoxyribonucleotide triphosphates and primers were removed by using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The purified PCR product was stored at –20°C until used.

DNA Sequencing

PCR amplicons were sequenced by using the DTS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions and the sequencing primers listed in online Technical Appendix Table 2. The sequencing reactions were run on a CEQ8000 Automated Sequencer (Beckman Coulter) according to the manufacturer's instructions. Sequences determined in this study have been submitted to the EMBL/GenBank/DDBJ databases; accession numbers are shown in online Technical Appendix Table 1.

Phylogenetic Analyses

Total RNA was extracted from 81 FMD viruses in Ethiopia, and each VP1-coding region was successfully amplified by RT-PCR. The PCR products were directly sequenced on both strands to obtain the complete VP1 sequences, which were compared with the other relevant FMDV VP1 sequences within the same serotype (see Figures 1–5 for database accession numbers).

VP1 nucleotide sequences were aligned by using BioEdit 7.0.5.3 (*17*) and Clustal W (*18*). These alignments were used to construct distance matrices by using the Kimura 2-parameter nucleotide substitution model in the program MEGA 4.0 (*19*). Some previously published sequences of serotype O were incomplete at the 5′ end of the VP1 gene and consisted of 495 nucleotides rather than the full-length 639 nucleotides. Midpoint-rooted neighbor-joining trees were then constructed with MEGA 4.0 software. The robustness of the tree topology was assessed with 1,000 bootstrap replicates by using the model in MEGA 4.0. The serotype C sequences labeled PD-FMD in Figure 3 were supplied by the Project Directorate on FMD, Mukteswar, India (*20*).

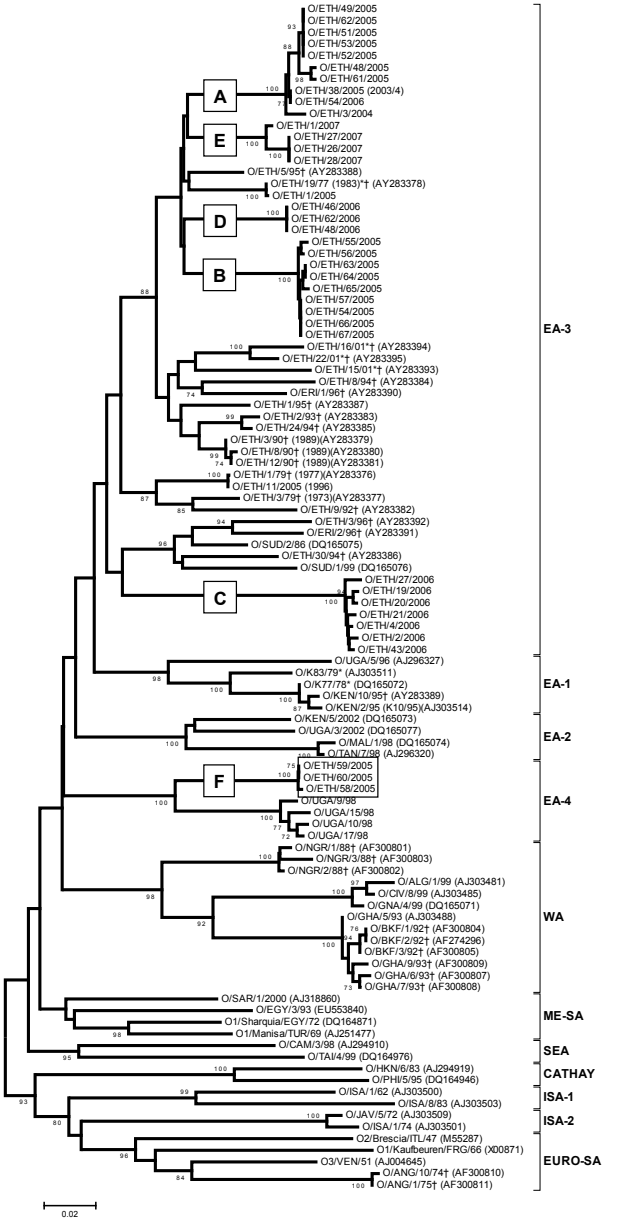

Figure 1. Midpoint-rooted neighbor-joining tree (based on the complete virus protein [VP] 1 coding sequence) showing the relationships between the foot-and-mouth disease virus serotype O isolates from Ethiopia and other contemporary and reference viruses. The 3 isolates from 2005 forming a new topotype East African (EA)-4 are boxed. The year in parenthesis indicates the year of sample collection. Scale bar indicates substitutions per site. *Not a reference number assigned by the World Reference Laboratory for Foot-and-Mouth Disease, Pirbright, UK. †Partial (495-nt) VP1 sequence used.

Vaccine Strain Selection

Vaccine strain selection for Ethiopian serotype O isolates was performed at WRLFMD by using the virus neutralization test. Relationship (r_1) values were determined as described elsewhere (*21*). An r_1 value of $\geq$0.3 was considered a good match with the vaccine strain (*22*).

Results

Distribution of FMD

FMD outbreaks occurred every year, but most (821) were reported in 1999 (Figure 6). This finding is consistent with previously reported findings (*10*,*23*) but is probably an underrepresentation of the actual situation. Of the 269 outbreak samples examined, FMDV was isolated from 82.2% (Table 1). During 1981–2007, a total of 5 serotypes (O, A, C, SAT 1, and SAT 2) were identified in bovine, swine, ovine, and caprine samples collected from the outbreak areas. FMDV O was the dominant serotype (73.3%), followed by types A (19.5%), SAT 2 (4.1%), SAT 1 (1.8%), and C (1.3%).

Geographically, the outbreaks were widely distributed. Most were within central Ethiopia, including the Addis Ababa administrative region; the rest were in Ahmara and Tigray in the north, Dire Dawa in the northeast, Beneshangul-Gumuz bordering Sudan in the west, and Southern Nations Nationalities and Peoples Region bordering Kenya and Sudan in the south (Figure 7). In eastern Ethiopia, poor veterinary services and inaccessibility to the area could have resulted in the lack of samples submitted.

In terms of species, cattle were found to be infected with all circulating serotypes of FMDV, whereas swine had only type O (Tables 1 and 2). SAT 2 was recorded in 2007, after an apparent gap of 16 years, from a bovine sample collected from Bambas, Beneshangul-Gumuz, western Ethiopia bordering Sudan (Figure 7, panel B). The first recorded occurrence of FMDV type SAT 1 in Ethiopia was identified from a bovine sample collected in November 2007 from the Mizan Teferi area bordering Kenya (Figure 7, panel B). Analysis of the samples collected from the same region 1 month later, in December 2007, showed involvement of 3 species: cattle, sheep, and goats.

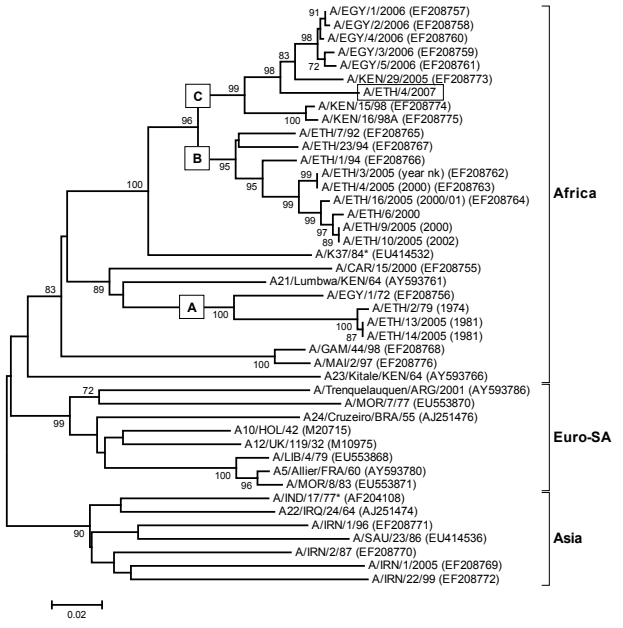

Figure 2. Midpoint-rooted neighbor-joining tree (based on the complete virus protein [VP] 1 coding sequence) showing the relationships between the foot-and-mouth disease virus serotype A isolates from Ethiopia and other contemporary and reference viruses. The isolate from 2007 is boxed. The year in parenthesis indicates the year of sample collection. Scale bar indicates substitutions per site. *Not a reference number assigned by the World Reference Laboratory for Foot-and-Mouth Disease, Pirbright, UK.

Phylogenetic Analyses

Serotype O

Of the FMDVs examined, serotype O predominated. All but 3 of the 55 FMDV serotype O Ethiopia isolates examined in our study fell into a single topotype, East Africa (EA)-3 (Figure 1). These 3 samples from 2005 were collected from cattle in the Mizan Teferi area (southwest of Addis Ababa) and formed a new serotype O topotype, which we named EA-4. Four viruses from Uganda in 1998 (≈91% nucleotide identity) also belonged to this topotype. The VP1 sequences of viruses within EA-4 differed by ≈14%–16% from members of the EA-1, EA-2, and EA-3 topotypes. Ethiopia type O viruses isolated during 2003–2007 fell into 6 lineages (A–F; Figure 1), which appeared to be cocirculating in different geographic regions (Figure 7, panel A; online Technical Appendix Table 1).

Serotype A

All viruses from Ethiopia belonged to the AFRICA topotype (Figure 2) (*6*). Three distinct lineages comprising viruses from Ethiopia were evident; all had high bootstrap support. Lineage A (1979 and 1981) also contained a single virus from Egypt in 1972; lineage B (1992–2002) contained only viruses from Ethiopia; and lineage C (2007) also contained viruses from Kenya (1998 and 2005) and Egypt (2006). Lineage B spanned 11 years and contained viruses isolated from 4 main regions—Oromiya, Tigray, Dire Dawa, and Southern Nations Nationalities and Peoples Region—indicating widespread dispersal of type A viruses (Figure 2). The virus isolated in 2007 (A/ETH/4/2007) was more closely related to the virus isolated from Kenya in 2005 (≈5% nt difference) than to that isolated from Ethiopia in 2000–2002 (≈10% nt difference).

Serotype C

Serotype C was not identified after 1983. Phylogenetic analysis showed that all serotype C viruses from Africa belonged to a single lineage (Figure 3), which has been designated the AFRICA topotype (N.J. Knowles, unpub. data). The 2 virus isolates from 1983 were closely related to a virus from Ethiopia in 1971 (≈98.5% nt identity) and grouped with the Kenya vaccine strain, K267/67 (≈94% nt identity) (Figure 3).

Serotypes SAT 1 and SAT 2

Genetic characterization of the newly identified SAT 1 isolates from Ethiopia indicates that they are all closely related but distinct from all other SAT 1 viruses from East Africa examined in this study (Figure 4). They were most closely related to viruses from Niger and Nigeria during 1975–1976 (topotype V) (*24*) but were different enough (SAT1/ETH/4/2007 vs. SAT1/NIG/11/75, 23% nt difference) to be classified as a new topotype, which we named topotype IX.

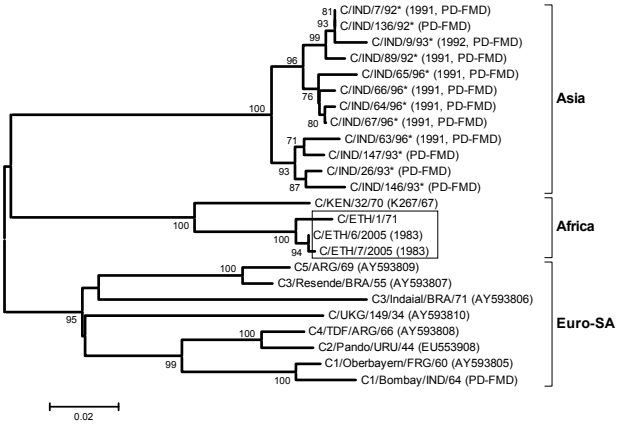

Figure 3. Midpoint-rooted neighbor-joining tree (based on the complete virus protein [VP] 1 coding sequence) showing the relationships between the foot-and-mouth disease virus serotype C isolates from Ethiopia (boxed) and other contemporary and reference viruses. The year in parenthesis indicates the year of sample collection. Scale bar indicates substitutions per site. *Not a reference number assigned by the World Reference Laboratory for Foot-and-Mouth Disease, Pirbright, UK.

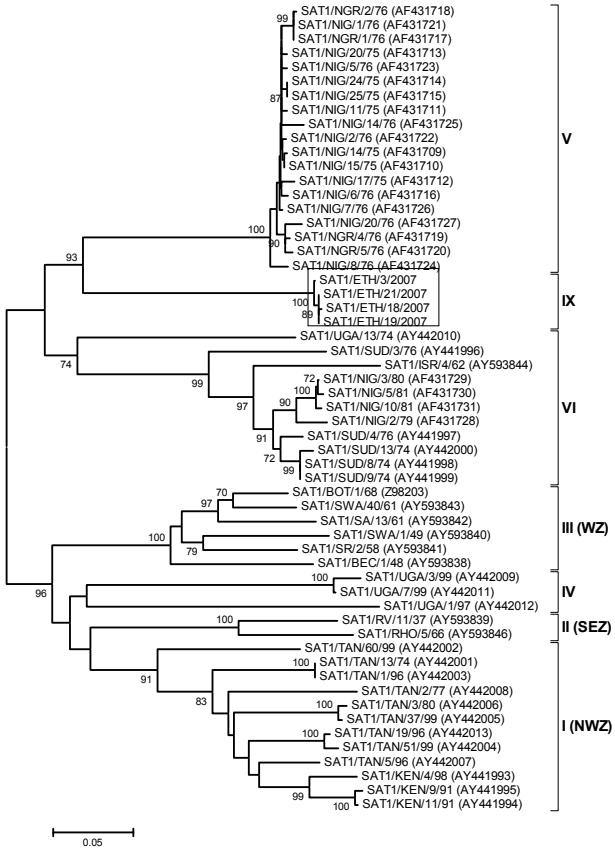

Figure 4. Midpoint-rooted neighbor-joining tree (based on the complete virus protein 1 coding sequence) showing the relationships between the foot-and-mouth disease virus serotype Southern African Territories (SAT) 1 isolates from Ethiopia and other contemporary and reference viruses. The 4 isolates from Ethiopia in 2007 are boxed. The year in parenthesis indicates the year of sample collection. Scale bar indicates substitutions per site. *Not a reference number assigned by the World Reference Laboratory for Foot-and-Mouth Disease, Pirbright, UK.

The first isolation of SAT 2 was in 1989 from a sample collected from cattle raised on Leben Ranch, Borena Zone, in southern Ethiopia (*9*); the virus was detected for the next 2 years but not again until 2007, an apparent gap of 16 years. Phylogenetic analysis of SAT 2 viruses from Ethiopia shows 3 distinct topotypes: IV (isolates from 1989), XIV (isolates from 1991), and XIII (single isolate from 2007) (Figure 5). Topotype IV has been detected in other African countries (Burundi, Malawi, Kenya, and Tanzania); topotype XIV was isolated only from Ethiopia. The new 2007 SAT 2 isolate from Beneshangul-Gumuz, Ethiopia (regional state bordering Sudan), did not group under either of the above-mentioned topotypes; rather, it could be assigned to topotype XIII along with Sudan isolates (SUD/6/77 and SUD/9/77, ≈81% nt identity) supported by a bootstrap value of 99% (Figure 5).

Vaccine Strain Selection

For vaccine strain selection for the new topotype of serotype O FMDV (EA-4), serologic tests were conducted. The extent of in vitro cross-neutralization of

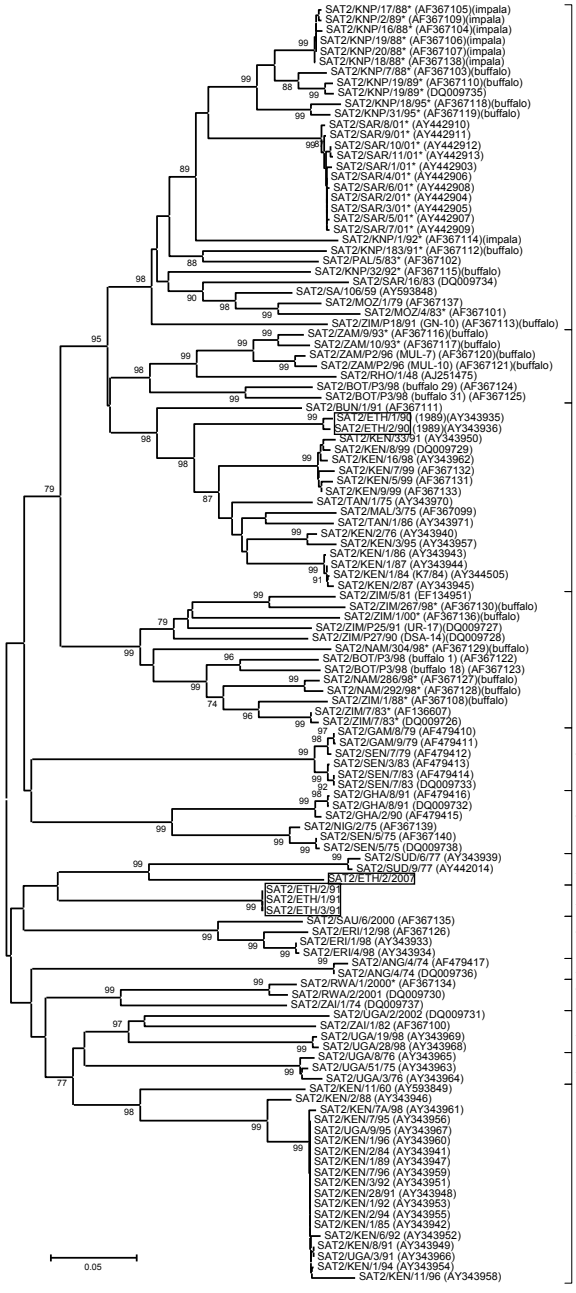

Figure 5. Midpoint-rooted neighbor-joining tree (based on the complete virus protein 1 coding sequence) showing the relationships between the foot-and-mouth disease virus serotype Southern African Territories (SAT) 2 isolates from Ethiopia and other contemporary and reference viruses. The SAT 2 isolates from Ethiopia under lineage IV, XIII, and IVX are boxed. The year in parenthesis indicates the year of sample collection. Scale bar indicates substitutions per site. *Not a reference number assigned by the World Reference Laboratory for Foot-and-Mouth Disease, Pirbright, UK.

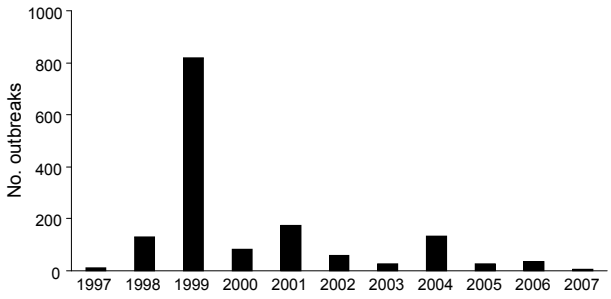

Figure 6. Number of foot-and-mouth disease outbreaks per year in different parts of the country, 1997–2007. Data from Ministry of Agriculture and Rural Development, Ethiopia; data for 1981–1996 not available.

O/ETH/58/2005 by antiserum against serotype O vaccine strains was evaluated. The match against vaccine strains O_1 Manisa and O_1 Lausanne were above the cutoff value of 0.3 (r_1 0.42 and 0.32, respectively), which indicated that both strains can confer protection for the new topotype (*22*). Similarly, representative samples from topotype EA-3 (O/ETH/9/2005 and O/ETH/10/2005) showed the O_1 Manisa vaccine strain to be the vaccine of choice (r_1 0.63 and 0.52, respectively).

Discussion

Similar to our findings, previous studies also have indicated serotype O to be highly prevalent and a dominant serotype causing most of the outbreaks in Ethiopia (*7*,*8*,*23*). The molecular epidemiology of serotype O has been well studied (*6*,*12*). Our study showed the existence of a fourth FMDV serotype O EA topotype. On the basis of comparison of sequence data of the VP1 gene, existence of 8 serotype O topotypes has been demonstrated within samples collected around the world (*12*). Among those, 2 topotypes were found in Africa, 1 in East Africa, and 1 in West Africa. Sangare et al. (*25*) described 7 genotypes of serotype O virus, 4 of which contained isolates from Africa. Samuel and Knowles (*12*) reported that isolates from Kenya and Uganda formed part of a single East African topotype (EA); viruses from Ethiopia, Tanzania, and Eritrea belonged to the Middle East–South Asia (ME-SA) topotype. However, their study was based on partial VP1 sequence data (3′ end of the gene), and it has been suggested that the relationships observed may have resulted from previous recombination events (*26*). Knowles et al. (*26*) renamed the EA topotype as EA-1 and identified 2 additional EA topotypes: EA-2 in Burundi, Kenya, Malawi, Rwanda, Tanzania, Uganda, and northern Zambia and EA-3 in Eritrea, Ethiopia, and Sudan.

Since 1983, serotype C seems to have disappeared from Ethiopia (Figure 3). However, a recent report of serotype C–specific antibodies in cattle in Ethiopia (*27*) indicates that circulation of serotype C viruses in Ethiopia may have gone unnoticed. No outbreaks of serotype C have been reported in Europe since 1989 (Italy), in South America since 2004 (Brazil), in Asia since 1995 (India and the Philippines) or 1996 (Nepal), and in Africa since 2004 (Kenya) (*28*). Therefore, investigations of the epidemiology of serotype C viruses in Ethiopia are urgently needed.

Genetic characterization of SAT type viruses is well documented for the southern African region; emphasis has been on viruses isolated from the African buffalo (*Syncerus caffer*) (*29*–*32*) and on cattle viruses from West Africa (*33*) and East Africa (*24*). On the basis of nucleotide sequence analysis of a portion of the viral genomes obtained from buffalo and domestic animals in sub-Saharan Africa, 14 independently evolving viral genotypes were identified for SAT 2, 8 for SAT 1, and 6 for SAT 3 (*5*). FMDV SAT 1 was first isolated in Ethiopia (Bench Maji, Southern Nations Nationalities and Peoples Region) in 2007

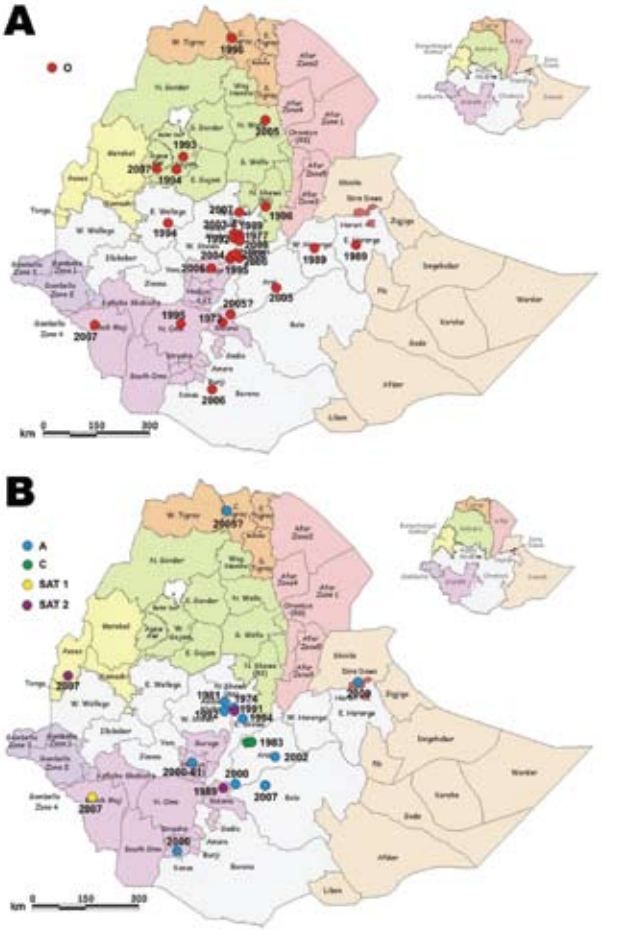

Figure 7. Location of cases of various foot-and-mouth disease (FMD) virus serotypes in the outbreaks of FMD, Ethiopia, 1981–2007, as evidenced by laboratory diagnosis. A) Serotype O, B) serotypes A, C, Southern African Territories (SAT) 1, and SAT 2. All boundaries are approximate and unofficial. Original map produced by United Nations Emergencies Unit for Ethiopia, 2000.

Table 1. Species of animals affected and foot-and-mouth disease virus serotypes identified in outbreaks in Ethiopia, 1981–2007*

Species	No. samples tested/no. positive†	Serotype				
		O	A	C	SAT 1	SAT 2
Bovine	250/216	159	43	3	2	9
Swine	7/3	3	–	–	–	–
Sheep	5/1	–	–	–	1	–
Goats	7/1	–	–	–	1	–
Total	269/221	162	43	3	4	9

*SAT, Southern African Territories; –, not identified.
†Positive results determined by cytopathic effect.

from samples collected from 3 species: cattle, sheep, and goats. Although SAT 1 has not been previously reported in Ethiopia (*7*,*8*,*24*), it might be circulating within wildlife and infrequently transmitted to domestic animals. SAT 2 may have been recently introduced by animal movement across the border with Sudan because SAT 2 is endemic to Sudan (*5*,*34*). Recent presence of serotype SAT 2–specific antibodies in cattle in Ethiopia has been reported (*27*). Two explanations are possible: 1) the virus is present in Ethiopia but has not been detected because all outbreaks are not reported or investigated, or 2) type SAT 2 viruses circulate subclinically in Ethiopia, possibly in wildlife.

Table 2. Serotypes of foot-and-mouth disease viruses isolated in Ethiopia, 1981–2007*

Year	Serotype					Total
	O	A	C	SAT 1	SAT 2	
1981	–	2	–	–	–	2
1982	10	8	–	–	–	18
1983	4	–	3	–	–	7
1984	–	7	–	–	–	7
1985	–	7	–	–	–	7
1986	2	2	–	–	–	4
1987	6	–	–	–	–	6
1988	3	–	–	–	–	3
1989	3	–	–	–	2	5
1990	15	–	–	–	2	17
1991	2	–	–	–	4	6
1992	12	1	–	–	–	13
1993	4	–	–	–	–	4
1994	10	12	–	–	–	22
1995	5	–	–	–	–	5
1996	1	1	–	–	–	2
1997†	–	–	–	–	–	0
1998	10	–	–	–	–	10
1999	16	–	–	–	–	16
2000	3	1	–	–	–	4
2001	12	–	–	–	–	12
2002	–	1	–	–	–	1
2003	3	–	–	–	–	3
2004	26	–	–	–	–	26
2005	4	–	–	–	–	4
2006	7	–	–	–	–	7
2007	4	1		4	1	10
Total	162	43	3	4	9	221

*SAT, Southern African Territories; –, not isolated.
†Samples not collected or not received.

FMD is endemic to Ethiopia as it is in all the bordering countries—Eritrea in the northeast, Sudan in the west, Kenya in the south, and Somalia in the east—and restriction of animal movement is limited. A large number of wildlife, including African buffalo (particularly in the Mago and Omo national parks), could act as FMDV reservoirs. The association of SAT serotypes with wildlife, particularly African buffalo, has been indicated (*5*,*24*,*35*,*36*). Individual buffalo can harbor the virus for as long as 5 years, and an isolated buffalo herd can maintain FMDV for 24 years (*37*). Transmission of virus from infected buffalo to other susceptible animals in close contact has been demonstrated (*38–40*). Therefore, transmission of FMDV by cattle movement or from wild animals to domestic animals is likely and may play a role in FMD outbreaks and in the appearance of new topotypes in Ethiopia.

The veterinary infrastructure for FMD disease surveillance and also the outbreak reporting system, which have not been efficient because of things such as financial constraints and difficulty accessing some regions, has improved considerably since the 1990s. Hence, this study may not be a true reflection of the number of serotypes/topotypes present in Ethiopia because not all outbreaks are reported or investigated due to the endemic nature of the disease. Therefore, comprehensive studies, including wildlife for molecular epidemiology and representative samples from all regions, are needed.

In terms of selecting vaccine strains, assessing the threat of SAT 1 and SAT 2 viruses in Ethiopia is difficult because SAT viruses often appear sporadically and then disappear in Ethiopia (only 1 SAT 1 and 4 SAT 2 samples have been isolated in 2007, and to date, no SAT outbreak has been reported in Ethiopia). Therefore, regular monitoring of the circulation of these viruses in livestock may help with selection of appropriate vaccine strains for FMD control.

In conclusion, the epidemiology of FMD in Ethiopia is complex because multiple serotypes of the virus (O, A, SAT 1, and SAT 2) circulate, 4 host species (cattle, sheep, goats, and pig) are involved, and high numbers of wildlife (especially African buffalo) cross the borders of neighboring countries uncontrolled. In addition, lack of prophylactic vaccination and veterinary infrastructure to handle outbreaks on a large scale greatly contribute to the frequent occurrence of the disease and make control of FMD extremely challenging. Regular monitoring and more detailed investigation are needed to formulate an efficient vaccine-based FMD control strategy for Ethiopia.

Acknowledgments

We thank the Agricultural Research Fund (ARFCG-2003) program, Ethiopian Agriculture Research Institute, for funding this project; the Ministry of Agriculture and Rural Development,

Ethiopia, for making the outbreak data available for the study; Robert Statham for conducting vaccine strain differentiation studies; and David Paton and Donald King for critically reading the manuscript.

An 8-month visit by G.A. to the Institute for Animal Health was supported by Rothamsted International African Fellowship Programme. Molecular analyses were supported by the Department for Environment, Food and Rural Affairs, UK (grant numbers SE2921, SE2935, and SE2937).

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References

1. Mattion N, König G, Seki C, Smitsaart E, Maradei E, Robiolo B, et al. Reintroduction of foot-and-mouth disease in Argentina: characterisation of the isolates and development of tools for the control and eradication of the disease. Vaccine. 2004;22:4149–62. DOI: 10.1016/j.vaccine.2004.06.040
2. Brooksby JB. Portraits of viruses: foot-and-mouth disease virus. Intervirology. 1982;18:1–23. DOI: 10.1159/000149299
3. Cartwright B, Chapman WG, Sharpe RT. Stimulation by heterotypic antigens of foot-and-mouth-disease virus antibodies in vaccinated cattle. Res Vet Sci. 1982;32:338–42.
4. Sahle M, Venter EH, Dwarka RM, Vosloo W. Molecular epidemiology of serotype O foot-and-mouth disease virus isolated from cattle in Ethiopia between 1979–2001. Onderstepoort J Vet Res. 2004;71:129–38.
5. Vosloo W, Bastos ADS, Sangare O, Hargreaves SK, Thomson GR. Review of the status and control of foot and mouth disease in sub-Saharan Africa. Rev Sci Tech Off Int Epizoot. 2002;21:437–49.
6. Knowles NJ, Samuel AR. Molecular epidemiology of foot-and-mouth disease virus. Virus Res. 2003;91:65–80. DOI: 10.1016/S0168-1702(02)00260-5
7. Martel JL. Foot-and-mouth disease in Ethiopia. Distribution of serotypes of foot-and-mouth disease virus [in French]. Rev Elev Med Vet Pays Trop. 1974;27:169–75.
8. Martel JL. Comparative serological study of the principal strains of the foot and mouth disease virus isolated in Ethiopia 1969–1974. Rev Elev Med Vet Pays Trop. 1975;28:287–95.
9. Roeder PL, Abraham G, Mebratu GY, Kitching RP. Foot-and-mouth disease in Ethiopia from 1988 to 1991. Trop Anim Health Prod. 1994;26:163–7. DOI: 10.1007/BF02241075
10. Asfaw W. Sintaro T. The status of FMD in Ethiopia, a growing concern. Ethiopian Veterinary Epidemiology. 2000;1:1–5.
11. Megersa B, Beyene B, Abunna F, Regassa A, Amenu K, Rufael T. Risk factors for foot and mouth disease seroprevalence in indigenous cattle in southern Ethiopia: the effect of production system. Trop Anim Health Prod. 2009 Aug;41:891–8. Epub 2008 Dec 4.
12. Samuel AR, Knowles NJ. Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). J Gen Virol. 2001;82:609–21.
13. World Organisation for Animal Health. OIE manual of standards for diagnostic tests and vaccines. 6th ed. Paris: The Organisation; 2008. p. 190–216.
14. Kitching RP, Rendle R, Ferris NP. Rapid correlation between field isolates and vaccine strains of foot-and-mouth disease virus. Vaccine 1988;6:403–8. DOI: 10.1016/0264-410X(88)90139-9
15. Roeder PL, Le Blanc Smith PM. The detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. Res Vet Sci. 1987;43:225–32.
16. Knowles NJ, Samuel AR, Davies PR, Midgley RJ, Valarcher JF. Pandemic strain of foot-and-mouth disease virus serotype O. Emerg Infect Dis. 2005;11:1887–93.
17. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8 [cited 2009 Jul 28]. Available from http://www.mbio.ncsu.edu/JWB/papers/1999Hall1.pdf
18. 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
19. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
20. Hemadri D, Sanyal A, Tosh C, Venkataramanan R, Pattnaik B. Serotype C foot-and-mouth disease virus isolates from India belong to a separate so far not described lineage. Vet Microbiol. 2003;92:25–35. DOI: 10.1016/S0378-1135(02)00354-1
21. Rweyemamu MM, Pay TWF, Parker MJ. Serological differentiation of foot-and-mouth disease virus strains in relation to selection of suitable vaccine viruses. Dev Biol Stand. 1976;35:205–4.
22. Paton DJ, Valarcher JF, Bergmann I, Matlho OG, Zakharov VM, Palma EL, et al. Selection of foot and mouth disease vaccine strains: a review. Rev Sci Tech. 2005;24:981–93.
23. Gelaye E, Beyene B, Ayelet G. Foot and mouth disease virus serotype identified in Ethiopia. Ethiopian Veterinary Journal. 2005;9:75–9.
24. Sahle M, Dwarka RM, Venter EH, Vosloo W. Comparison of SAT-1 foot-and-mouth disease virus isolates obtained from East Africa between 1971 and 2000 with viruses from the rest of sub-Saharan Africa. Arch Virol. 2007;152:797–804. DOI: 10.1007/s00705-006-0893-x
25. Sangare O, Bastos ADS, Marquardt O, Venter EH, Vosloo W, Thomson GR. Molecular epidemiology of serotype O foot-and-mouth disease virus with emphasis on West and South Africa. Virus Genes. 2001;22:345–51. DOI: 10.1023/A:1011178626292
26. Knowles NJ, Davies PR, Midgley RJ, Valarcher J-F. Identification of a ninth foot-and-mouth disease virus type O topotype and evidence for a recombination event in its evolution. In: Report of the Session of the Research Group of the Standing Technical Committee of EUFMD; 2004 Oct 12–15; Chania, Crete, Greece. p. 163–72.
27. Rufael T, Catley A, Bogale A, Sahle M, Shiferaw Y. Foot and mouth disease in the Borana pastoral system, southern Ethiopia and implications for livelihoods and international trade. Trop Anim Health Prod. 2008;40:29–38. DOI: 10.1007/s11250-007-9049-6
28. Roeder PL, Knowles NJ. Foot-and-mouth disease virus type C situation: the first target for eradication? In: Report of the Session of the Research Group of the Standing Technical Committee of EUFMD; 2008 Oct 14–17; Erice, Sicily, Italy. In press.
29. Vosloo W, Kirkbride E, Bengis RG, Keet DF, Thomson GR. Genome variation in the SAT types of foot-and-mouth disease viruses prevalent in buffalo (*Syncerus caffer*) in the Kruger National Park and other regions of southern Africa, 1986–93. Epidemiol Infect. 1995;114:203–18. DOI: 10.1017/S0950268800052055
30. Vosloo W, Knowles NJ, Thomson GR. Genetic-relationships between southern African SAT-2 isolates of foot-and-mouth disease virus. Epidemiol Infect. 1992;109:547–58. DOI: 10.1017/S0950268800050536
31. Bastos ADS, Haydon DT, Forsberg R, Knowles NJ, Anderson EC, Bengis RG, et al. Genetic heterogeneity of SAT-1 type foot-and-mouth disease viruses in southern Africa. Arch Virol. 2001;146:1537–51. DOI: 10.1007/s007050170077

32. Bastos ADS, Haydon DT, Sangare O, Boshoff CI, Edrich JL, Thomson GR. The implications of virus diversity within the SAT 2 serotype for control of foot-and-mouth disease in sub-Saharan Africa. J Gen Virol. 2003;84:1595–606. DOI: 10.1099/vir.0.18859-0
33. Sangare O, Bastos ADS, Venter EH, Vosloo W. Retrospective genetic analysis of SAT-1 type foot-and-mouth disease outbreaks in West Africa (1975–1981). Vet Microbiol. 2003;93:279–89. DOI: 10.1016/S0378-1135(02)00439-X
34. World Organisation for Animal Health. World animal health in 2001. Reports on the animal health status and disease control methods. Paris: The Organisation; 2002. p. 131–2.
35. Bastos ADS, Boshoff CI, Keet DF, Bengis RG, Thomson GR. Natural transmission of foot-and-mouth disease virus between African buffalo (*Syncerus caffer*) and impala (*Aepyceros melampus*) in the Kruger National Park, South Africa. Epidemiol Infect. 2000;124:591–8. DOI: 10.1017/S0950268899004008
36. Thomson GR. The role of carrier animals in the transmission of foot and mouth disease. Comprehensive reports on technical items presented to the international committee or to regional commissions. Paris: World Organisation for Animal Health; 1997. p. 87–103.
37. Condy JB, Hedger RS, Hamblin C, Barnett ITR. The duration of the foot-and-mouth-disease virus carrier state in African buffalo (i) in the individual animal and (ii) in a free-living herd. Comp Immunol Microbiol Infect Dis. 1985;8:259–65. DOI: 10.1016/0147-9571(85)90004-9
38. Dawe PS, Flanagan FO, Madekurozwa RL, Sorensen KJ, Anderson EC, Foggin CM, et al. Natural transmission of foot-and-mouth-disease virus from African buffalo (*Syncerus caffer*) to cattle in a wildlife area of Zimbabwe. Vet Rec. 1994;134:230–2.
39. Dawe PS, Sorensen K, Ferris NP, Barnett ITR, Armstrong RM, Knowles NJ. Experimental transmission of foot-and-mouth-disease virus from carrier African buffalo (*Syncerus caffer*) to cattle in Zimbabwe. Vet Rec. 1994;134:211–5.
40. Vosloo W, Bastos AD, Kirkbride E, Esterhuysen DJ, vanRensburg DJ, Bengis RG, et al. Persistent infection of African buffalo (*Syncerus caffer*) with SAT-type foot-and-mouth disease viruses: rate of fixation of mutations, antigenic change and interspecies transmission. J Gen Virol. 1996;77:1457–67. DOI: 10.1099/0022-1317-77-7-1457

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Clinical and Epidemiologic Characteristics of 3 Early Cases of Influenza A Pandemic (H1N1) 2009 Virus Infection, People's Republic of China, 2009

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On May 7, 2009, a national network was organized in the People's Republic of China for the surveillance, reporting, diagnosis, and treatment of influenza A pandemic (H1N1) 2009 virus infection (pandemic [H1N1] 2009). Persons with suspected cases are required to report to the Chinese Center for Disease Control and Prevention and the Ministry of Health within 24 hours; the patient's close contacts are then traced and placed in quarantine for 7 days. We report 3 confirmed early cases of pandemic (H1N1) 2009. Two cases were imported from United States; the other was imported from Canada. The patients exhibited fever and signs and other symptoms that were indistinguishable from those of seasonal influenza. Serial virologic monitoring of pharyngeal swabs showed that they were negative for pandemic (H1N1) 2009 virus by real-time reverse transcription–PCR 4–6 days after onset of illness. One close contact whose sample tested positive for pandemic (H1N1) 2009 virus had no symptoms during quarantine. A national network is essential for controlling pandemic (H1N1) 2009.

In early April 2009, human infections caused by an influenza virus designated as influenza A pandemic (H1N1) 2009 virus (pandemic [H1N1] 2009) were identified in United States (*1*,*2*) and Mexico (*3*) and spread rapidly to other regions of the world (*4*,*5*). Pandemic (H1N1) 2009 virus has been reported to be a triple reassortant influenza virus (containing genes from human, porcine, and avian influenza viruses) that has circulated among swine in the United States since 1999 (*6*–*8*). Sporadic human infections by influenza virus of swine origin had been described, mostly in young persons in contact with pigs (*9*). The current outbreak indicates that the new pandemic (H1N1) 2009 virus can be transmitted from human to human. As of June 17, 2009, a total of 88 countries officially reported a cumulative total of 39,620 laboratory-confirmed pandemic (H1N1) 2009 cases; these cases occurred in Mexico (6,241 cases [including 108 deaths]), the United States (17,855 [44 deaths]), Canada (4,049 [7 deaths]), Chile (2,335 [2 deaths]), Argentina (733 [1 death]), the United Kingdom (1,461 [1 death]), Costa Rica (149 [1 death]), Australia

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DOI: 10.3201/eid1509.090794

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(2,112), Japan (666), the People's Republic of China (382), Germany (195), France (118), and other countries (*10*). In this report, we describe the clinical and epidemiologic characteristics of 3 early confirmed cases of pandemic (H1N1) 2009 in China.

Methods

Surveillance, Reporting, and Data Collection

On May 7, 2009, a national network for monitoring pandemic (H1N1) 2009 was organized in China. The partners included the Ministry of Health (MOH), the Chinese Center for Disease Control and Prevention (Chinese CDC), and all community hospitals and teaching hospitals. A guideline for the surveillance, reporting, diagnosis, and treatment of pandemic (H1N1) 2009 was published on May 9, 2009. Any ill persons with a body temperature ≥37.5°C were asked to visit the fever clinics in local general hospitals.

A confirmed case of human infection with pandemic (H1N1) 2009 virus was defined as laboratory confirmation of infection from a human sample at the Chinese CDC or the Chinese Academy of Medical Sciences (CAMS) (see the Laboratory Confirmation section below). A suspected case was defined as 1) an influenza-like illness (ILI; fever ≥37.5°C with at least 1 symptom or sign, including sore throat, cough, rhinorrhea, nasal congestion) in a person who has traveled to a country where ≥1 confirmed pandemic (H1N1) 2009 cases had been found in the past 7 days, or 2) clinical symptoms or signs of ILI in a person with an epidemiologic link to a patient found to have confirmed or suspected pandemic (H1N1) 2009 in the previous 7 days.

Close contacts were defined as persons who had lived with a person with probable or confirmed pandemic (H1N1) 2009 or who had had direct contact with the respiratory secretions or body fluids of such persons. All close contacts were quarantined for medical observation for 7 days; during this time, pharyngeal swabs were collected for identification of pandemic (H1N1) 2009 virus (once if results were negative; serial repeat tests if results were positive).

All suspected cases were required to be reported to the Chinese CDC and to the MOH within 24 hours of diagnosis. Pharyngeal swabs were forwarded to a local branch of the Chinese CDC for detection of influenza A pandemic (H1N1) 2009 virus by real-time reverse transcription–PCR (RT-PCR). All patients with a positive RT-PCR result for pandemic (H1N1) 2009 virus were admitted to the local infectious disease hospital where the patient was placed in isolation. Additional specimens were sent to the State Reference Influenza Laboratories at the Chinese CDC or CAMS for further characterization and nucleic acid sequencing.

A standardized surveillance reporting form was used to collect clinical, epidemiologic, or demographic data. We included information about the patient's demographic characteristics, underlying medical conditions, status with respect to seasonal influenza vaccination, exposures to swine and other animals, travel to a country with confirmed pandemic (H1N1) 2009 infection, clinical signs and symptoms, chest radiograph results, laboratory findings, results of diagnostic testing for influenza, antiviral treatment, clinical complications, and clinical outcome.

Laboratory Confirmation

Pharyngeal swabs were collected from all patients and their close contacts, which were submitted to local branch of the Chinese CDC and reference laboratories in the Chinese CDC or CAMS for investigation. We used the protocol of the US Centers for Disease Control and Prevention of real-time RT-PCR for pandemic (H1N1) 2009 as recommended by the World Health Organization (*11*). The PCR products were sequenced for further confirmation by standard high-throughput sequencing system with the use of BigDye Terminator, version 3.1 (Applied Biosystems, Foster City, CA, USA) with 1 mm^3 of double-stranded template.

Results

The demographic and epidemiologic characteristics of the 3 patients with confirmed pandemic (H1N1) 2009, including the estimated disease incubation period and their travel histories, are listed in Table 1. Their clinical characteristics are shown in Table 2. All 3 patients were Chinese students who had been studying abroad (2 in United States and 1 in Canada).

Patient 1 left St. Louis, Missouri, USA, on May 7, 2009 (US date), and flew to St. Paul, Minnesota, USA, where he transferred to a flight to Tokyo, Japan, then to

Table 1. Demographic and epidemiologic characteristics of 3 patients infected with influenza A pandemic (H1N1) 2009 virus, People's Republic of China, 2009

Characteristic	Patient 1	Patient 2	Patient 3
Age, y	30	19	18
Sex	M	M	F
City of origin	St. Louis, MO, USA	Winnipeg, Manitoba, Canada	New York, NY, USA
Date of illness onset	May 9	May 10	May 13
Site of illness onset	Airplane	Hotel	Home
City of illness onset	Beijing	Beijing	Beijing
City of virus isolation	Chengdu	Jinan	Beijing

Beijing, China, arriving on May 8 (Beijing date). The patient did not experience illness, and fever did not develop during the flight from St. Louis to Beijing. At the Beijing airport, his temperature was normal. The patient stayed in Beijing for 1 night and boarded a flight to Chengdu on May 9. He did not report feeling ill before the flight. On the flight from Beijing to Chengdu, fever developed. Upon landing, the patient went to a hospital for medical attention for his illness. The case was reported to the Chinese CDC, and specimens were collected on May 9. A test result for pandemic (H1N1) 2009 virus was positive on May 10 by real-time RT-PCR. All 144 close contacts (including his father, girlfriend, taxi driver, and all passengers on the flight from Chendu to Beijing) were quarantined in hotels for medical observation for 7 days.

Patient 2 left Winnipeg, Manitoba, Canada, on May 7 (Canada date) and arrived in Beijing on May 8 (Beijing date). He did not feel ill while traveling from Winnipeg to Beijing. He stayed for 2 days in Beijing, where a fever developed, but he did not seek medical attention. The patient subsequently took a train from Beijing to Jinan on May 11 and, on arrival, went to the local infectious diseases hospital. There was no thermal scanner for fever at the train station in Beijing. The case was reported to the Chinese CDC, and specimens were collected on May 11. The pandemic (H1N1) 2009 virus test result was positive on May 12 by real-time RT-PCR. All 40 close contacts sitting in the same car of the train were quarantined for medical observation for 7 days.

Patient 3 left New York, New York, USA, on May 10 (US date) and arrived at home in Beijing on May 11 (Beijing date). She was well when traveling from New York to Beijing. Her temperature was normal at border health/temperature monitoring. She stayed home with her mother and did not meet other persons and visit other places. A fever developed on May 13 (Beijing date), and she sought medical attention on the evening of May 14. The case was reported to Chinese CDC, and specimens were collected on May 15. The test result for pandemic (H1N1) 2009 virus was positive on May 15 by real-time RT-PCR. Her mother and a taxi driver were the only close contacts.

All 3 patients were positive for pandemic (H1N1) 2009 virus by real-time RT-PCR, and further confirmed PCR products sequencing the partial hemagglutinin sequence from all 3 patients were 99.5–100% homologous with A/California/4/2009 but only 79.4%–79.6% homologous with seasonal influenza virus (H1N1) (A/Brisbane/59/2007) (Table 3). The dynamic virologic monitoring of pharyngeal swab samples from the 3 case-patients showed that real-time RT-PCR results for influenza A pandemic (H1N1) 2009 virus were negative on day 5, day 7, and day 6, respectively (Table 3).

Only 1 sample from a total of 186 close contacts tested positive for pandemic (H1N1) 2009 virus by real-time RT-PCR. This sample came from the 48-year-old mother of patient 3, who had lived with patient 3 for 2 days before her illness and had taken care of her for 2 days after her illness began. On the fifth day (May 16) after exposure to patient 3, the woman's sample became positive (Table 3). As with all the other close contacts, this woman had no fever or ILI symptoms.

Table 2. Clinical characteristics of 3 patients infected with influenza A pandemic (H1N1) 2009 virus, People's Republic of China, 2009*

Characteristic	Patient 1	Patient 2	Patient 3
Chronic illness	No	No	No
Influenza vaccine in last flu season	No	No	No
Highest temperature, °C	38.8	39	39.4
Sore throat	Yes	Yes	Yes
Cough	Yes	No	Yes
Rhinorrhea	Yes	No	Yes
Nasal congestion	Yes	No	No
Headache	Yes	Yes	Yes
Diarrhea	No	No	No
Other symptoms	No	Decreased appetite	Chest pain
Leukocyte count, per mm^3	7,900	4,900	5,300
Neutrophil count, per mm^3	5,480	2,810	4,000
Lymphocyte count, per mm^3	1,540	1,580	1,000
Platelet count, per mm^3	166,000	208,000	254,000
C-reactive protein, mg/L	39	Not done	0.3
Findings on chest radiograph	Normal	Normal	Normal
Oseltamivir treatment*	Yes	Yes	Yes
Duration of fever, d	2	3	3
Length of stay in hospital, d†	8	8	8
Outcome	Recovered	Recovered	Recovered

*75 mg 2x/d for 5 d.
†During their stay in hospital, no patient needed oxygen or a ventilator. They were kept in hospitals for isolation and close observation because pandemic (H1N1) 2009 was an infectious disease new to physicians in China.

Table 3. Dynamic virologic monitoring of influenza A pandemic (H1N1) 2009 virus by real-time RT-PCR of 3 patients and the mother of patient 3, People's Republic of China, 2009*

Day no.	Patient 1	Patient 2	Patient 3	Mother of patient 3
1	+ (May 9)	ND (May 10)	ND (May 13)	ND (May 13)
2	+	+	ND	ND
3	+	+	–	ND
4	+	+	+	ND (patient 3 isolated)
5	–	+	+	+
6	–	+	–	+
7	–	–	–	–
8	Discharged	–	–	–
9		Discharged	–	–
10			Discharged	–

*Day 1 of patients 1, 2, and 3 indicates the day of onset of fever. Day 1 of the mother of patient 3 indicates the day when fever developed in patient 3. Gray shading indicates days of oseltamivir treatment. RT-PCR, reverse transcription–PCR; ND: not done; +, positive for influenza A pandemic (H1N1) 2009 virus; –, negative for influenza A pandemic (H1N1) 2009 virus.

Discussion

The 3 cases were mild, and the patients were young, which is consistent with the profile of other pandemic (H1N1) 2009 infections reported around the world (*6*). The most frequent signs and symptoms in the patients were fever and other manifestations that were nonspecific for pandemic (H1N1) 2009 and indistinguishable from those of seasonal influenza. None of them had evidence of severe lower respiratory tract illness or unusual symptoms of influenza, such as diarrhea. All patients recovered quickly, with a median duration of fever of 3 days. All 3 patients had complete blood counts performed during the course of their disease, but none had leukopenia (leukocyte count <4,000/mm^3), or lymphopenia (total lymphocyte count <800/mm^3), or thrombocytopenia (total platelet count <100,000/mm^3).

The transmissibility of influenza A pandemic (H1N1) 2009 virus is uncertain. One study assumed that its transmissibility (R_0) is substantially higher than that of seasonal influenza and comparable with the viruses in previous influenza pandemics (*12*). Our study demonstrates, on the basis of PCR testing, that the average time of pandemic (H1N1) 2009 virus shedding is 4–6 days. Positive RT-PCR results do not necessarily confirm virus shedding, but the PCR-based method is more sensitive than culture methods for detecting virus shedding (*13*). Our study also shows that the risk for person-to-person transmission is greatest in households. Among 186 close contacts, only the mother of patient 3, who lived with her, had a positive result for pandemic (H1N1) 2009 virus by real-time RT-PCR, although she had no symptoms of influenza. All other close contacts, including taxi drivers, passengers in an airplane or the same car of a train, were negative for pandemic (H1N1) 2009 virus at screening, and illness did not develop subsequently while they were being observed. Asymptomatic infections appear to be possible among household members, as demonstrated by case-patient 3 and her mother.

Our study has several limitations. First, numbers in this series were low. Second, the exact date of exposure to a known infectious source was difficult to trace. Third, pharyngeal swab samples may have a lower sensitivity (than nasopharyngeal), and thus some false-negative results might have occurred, which could lead to underestimation of viral shedding.

A national network for the surveillance and control of pandemic (H1N1) 2009 was quickly organized in China, and the response was quick and thorough. The Chinese government is moving swiftly to contain the new influenza, drawing on lessons from the severe acute respiratory syndrome and bird influenza outbreaks in recent years. In all airports, thermal scanners have been installed, and all asymptomatic contacts of case-patients were quarantined for 7 days as part of the early response. As the number of imported cases has increased, the quarantine policy is changing. During the past 4 weeks, only symptomatic travelers at ports of entry and passengers sitting within a short distance (<2 m) to a person with a suspected or confirmed case were quarantined. As local transmission has been documented, more efforts have been paid to communities. Since June 22, hand temperature monitors were used in all schools in Beijing. All students with body temperatures >37.2°C were asked to stay at home until their temperature returned to normal. Further evaluation is needed to continue to clarify the nature of pandemic (H1N1) 2009, including its clinical features, severity, incubation period, and transmission patterns (*14*).

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References

1. Centers for Disease Control and Prevention. Swine-origin influenza A (H1N1) virus infections in a school—New York City, April 2009. MMWR Morb Mortal Wkly Rep. 2009;58:470–2.

2. Centers for Disease Control and Prevention. Swine influenza A (H1N1) infection in two children—Southern California, March–April 2009. MMWR Morb Mortal Wkly Rep. 2009;58:400–2.
3. Centers for Disease Control and Prevention. Outbreak of swine-origin influenza A (H1N1) virus infection—Mexico, March–April 2009. MMWR Morb Mortal Wkly Rep. 2009;58:467–70.
4. Centers for Disease Control and Prevention. Update: infections with a swine-origin influenza A (H1N1) virus—United States and other countries, April 28, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:431–3.
5. Naffakh N, van der Werf SV. An outbreak of swine-origin influenza A (H1N1) virus with evidence for human-to-human transmission. Microbes Infect. 2009;11:725–8.
6. Novel Swine-Origin Influenza A (H1N1 Virus Investigation Team. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009;360:2667–8.
7. Trifonov V, Khiabanian H, Greenbaum B, Rabadan R. The origin of the recent swine influenza A(H1N1) virus infecting humans. Euro Surveill. 2009;14:19193.
8. Centers for Disease Control and Prevention. Update: drug susceptibility of swine-origin influenza A (H1N1) viruses, April 2009. MMWR Morb Mortal Wkly Rep. 2009;58:433–5.
9. Shinde V, Bridges CB, Uyeki TM, Shu B, Balish A, Xu X, et al. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005–2009. N Engl J Med. 2009;360:2616–25.
10. World Health Organization. Influenza A (H1N1)—update 34. Geneva: The Organization; 2009 [cited 2009 May 18]. Available from http://www.who.int/csr/don/2009_05_20/en/index.html
11. Centers for Disease Control and Prevention (CDC). CDC protocol of real-time RT-PCR for swine influenza A (H1N1): World Health Organization, 28 April 2009 [cited 2009 May 18]. Available from www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf
12. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, et al. Pandemic potential of a strain of influenza A (H1N1): early findings. Science. 2009;324:1557–61. DOI: 10.1126/science.1176062.
13. Leekha S, Zitterkopf NL, Espy MJ, Smith TF, Thompson RL, Sampathkumar P. Duration of influenza A virus shedding in hospitalized patients and implications for infection control. Infect Control Hosp Epidemiol. 2007;28:1071–6. DOI: 10.1086/520101
14. Swine influenza: how much of a global threat? Lancet. 2009;373:1495. DOI: 10.1016/S0140-6736(09)60826-6

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Clinical Assessment and Improved Diagnosis of Bocavirus-induced Wheezing in Children, Finland

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Human bocavirus (HBoV) is a widespread respiratory virus. To improve diagnostic methods, we conducted immunoglobulin (Ig) G and IgM enzyme immunoassays with recombinant virus–like particles of HBoV as antigen. Acute-phase and follow-up serum samples from 258 wheezing children and single serum samples from 115 healthy adults in Finland were examined. Our assays had a sensitivity of 97% and a specificity of 99.5%. Of adults, 96% had immunity; none had an acute infection. Of 48 children with serologically diagnosed acute HBoV infections, 45 were viremic and 35 had virus in nasopharyngeal aspirates (NPAs). Of 39 HBoV NPA PCR–positive children co-infected with another virus, 64% had a serologically verified HBoV infection. HBoV caused illness of longer duration than rhinovirus and of equal severity to that of respiratory syncytial virus. Among children with bronchiolitis, >25% had acute HBoV infections. Accurate HBoV diagnosis requires serologic analysis or PCR of serum; PCR of NPAs alone is insufficient.

A new parvovirus, human bocavirus (HBoV), was discovered during sequencing of respiratory tract samples from children. It has been detected worldwide in the nasopharyngeal tract, mainly in small children with lower respiratory tract infections (*1,2*). HBoV has been associated with upper and lower respiratory tract infections and shown to be a cause of pneumonia in children (*3–8*). Prolonged shedding of virus has been reported; ≥26% of children shed virus for 2 months, 4% for 3 months, and 2% for 4 months (*9*). Diagnosis of HBoV respiratory tract infections has been PCR based, leading to overrepresentation of HBoV co-infections with other respiratory viruses (*9–11*).

Along with others, we have shown that respiratory infections with HBoV elicit B-cell immune responses (*11–15*) and can be diagnosed serologically by using prokaryotic virus protein 2 (VP2) antigens in immunoblots (*11*). We report production in insect cells of VP2 of virus-like particles (VLPs) and their use in enzyme immunoassays (EIAs) for detection of HBoV-specific immunoglobulin (Ig) M and IgG in paired serum samples of pediatric patients with acute wheezing and in single serum samples of young healthy adults. Serologic results were compared with those of HBoV quantitative PCR (qPCR) of nasopharyngeal aspirates (NPAs) and paired serum samples of 258 children with complete sample sets. Clinical signs and symptoms of wheezing children with serologically verified acute HBoV infections with or without other respiratory virus infections (15 other viruses studied [*10*]) were compared with those of children infected with respiratory syncytial virus (RSV) or rhinovirus.

Materials and Methods

Patients and Samples

Acute-phase (at the time of admission) and convalescent-phase (2 weeks later) serum samples and NPA samples at the time of admission were obtained from 259 children (age range 3 months to 15 years, median 1.6 years) with acute expiratory wheezing (*10,16*). These children were tested by NPA PCR for 16 respiratory viruses (*10*); 117 of these children were tested by HBoV IgM and IgG immunoblots and HBoV serum qPCR (*10,11*). All remaining serum samples, except 1 convalescent-phase serum sample that

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DOI: 10.3201/eid1509.090204

was depleted, were tested by HBoV qPCR specific for the nucleoprotein 1 gene as described (*11*); all serum samples were tested by EIA. For 93 of these 258 children, follow-up serum samples were obtained 5–8 years later. In addition, 115 serum samples from healthy medical students were collected after informed consent was obtained. The study was reviewed and approved by the Ethics Committees of Turku and Helsinki University hospitals.

Expression of VP2

The putative major virus capsid protein VP2 gene (nt 3443–5071) of the HBoV St 2 isolate (GenBank accession no. DQ000496) was cloned into a baculovirus vector pAcSG2 (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) by standard procedures and confirmed by sequencing. The VP2-containing vector was transfected into Sf9 insect cells by using FuGENE 6 Transfection reagent (Roche, Basel, Switzerland). Two million adherent cells in T25 bottles were transfected in 1 mL of Insect Express media (Lonza, Basel, Switzerland) with a mixture of 2 μg plasmid, 250 ng linearized baculoGold DNA (Becton Dickinson Biosciences), and 15 μL FuGENE reagent. Fresh cells were infected 3 times every third day by using virus medium collected from the previous infection. VP2-containing Sf9 cells were harvested on day 3, and cell pellets were resuspended in phosphate-buffered saline (PBS), pH 7.5, at a concentration of 2.1×10^7 cells/mL. Protease inhibitor (complete EDTA-free; Roche) was added (≈75 μL/mL), and cells were lysed by sonication (4 × 20 s). After subsequent centrifugation at 13,200 rpm for 3 min, VLPs were purified by 48-h CsCl gradient ultracentrifugation at 24,200 rpm (L-70 Ultracentrifuge; Beckman, Fullerton, CA, USA) at 4°C after fraction collection and dialysis against PBS. The product was concentrated in columns (Amicon Ultra-15 50,000 MWCO; Millipore, Billerica, MA, USA). Expressed HBoV VP2 was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to have a molecular mass of ≈60 kDa and by electron microscopy to be spherical symmetric parvovirus-like particles ≈20 nm in diameter (Figure 1). Before use as antigen, VLPs were biotinylated as described (*17*) .

Serologic Analysis

The HBoV IgG EIA was conducted as described for parvovirus B19 (*18*) except that biotinylated VLPs were used at concentrations of 60 ng/well. Cutoff values were calculated from IgG EIA absorbances of the first serum sample obtained at admission of 34 children who had no IgM or maternal (waning) IgG against HBoV and showed a ≥4-fold increase of IgG against HBoV during follow-up testing. Cutoff absorbances for negative and positive IgG EIA results were 0.154 (mean + 3 SD) and 0.188 (mean + 4 SD), respectively.

For IgM EIA, a μ-capture format was used (*19*). Serum samples diluted 1:200 in PBS and 0.05% Tween (PBST) were applied in duplicate into wells of plates coated with goat anti-human IgM (Cappel/ICN Biomedicals, Costa Mesa, CA, USA) for 60 min at room temperature. After being rinsed 5 times with PBST, biotinylated HBoV VLPs were applied at a concentration of 25 ng/well and incubated for 45 min at 37°C. Bound antigen was visualized by using horseradish peroxidase–conjugated streptavidin (Dako, Glostrup, Denmark) at 1:12,000 in PBST plus 0.5% bovine serum albumin for 45 min at 37°C, followed by *o*-phenylenediamine dihydrochloride (Dako) and H_2O_2 for 15 min at 37°C. Cutoff values were calculated from IgM EIA absorbances of 5-year follow-up samples of 61 children who were IgG positive 5 years earlier. Cutoff absorbances for negative and positive IgM EIA results were 0.136 (mean + 3 SD) and 0.167 (mean + 4 SD), respectively. Parvovirus B19 serologic analysis was conducted by using commercial (Biotrin, Dublin, Ireland) and in-house EIAs (*18,20*).

Statistical Analysis

Because most continuous data were skewed (by Kolmogorov-Smirnov test), they were analyzed by using regression analysis and generalized linear models after logarithmic transformation. Logistic regression analysis was used for categoric data. Statistical analyses were conducted by using SAS/STAT(r) software version 9.1.3 SP4 (SAS Institute Inc., Cary, NC, USA).

Results

qPCR

Complete sets of HBoV qPCR results for NPAs and serum samples were available for 258 children (Table 1). Among these children, 49 (19%) showed viremia in the first serum sample (29/49 [59%]), the second sample (12/49 [24%]), or both samples (8/49 [16%]). Most chil-

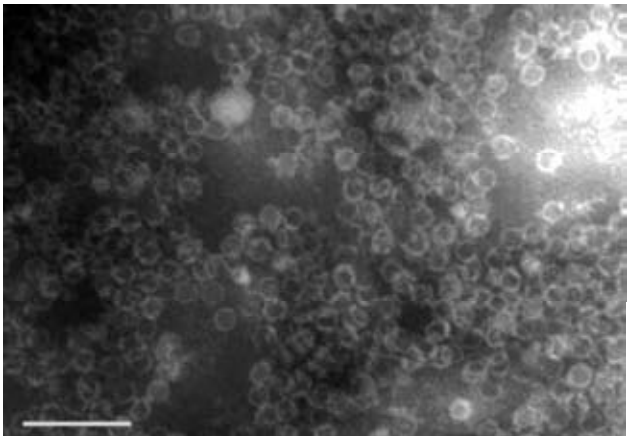

Figure 1. Recombinant human bocavirus virus protein 2 virus-like particles. Scale bar = 100 nm.

Table 1. Human bocavirus EIA and quantitative PCR results for 258 wheezing children, Finland*

PCR result	No. children	Serodiagnoses, IgM+,† no (%)					No serodiagnoses, no. (%)			
		SDG	SC	SI	IgG+	IgG–	All	IgG+	IgG–	Maternal
NPA+ serum+	34	33 (97)	21	4	6	2	1	0	1	0
NPA+ serum–	15	2 (13)	0	0	2	0	13	10	3	0
NPA– serum–	194	1† (0.5)	1†	0	0	0	193	100	88	5
NPA– serum+	15	12 (80)	6	1	2	3	3	1	2	0
NPA+	49	35 (71)	21	4	8	2	14	10	4	0
NPA+, high load	28	27 (96)	21	3	2	1	1	0	1	0
NPA+, low load	21	8 (38)	0	1	6	1	13	10	3	0
Serum+	49	45 (92)	27	5	8	5	4	1	3	0
NPA–	209	13 (6)	7†	1	2	3	196	101	90	5
Serum–	209	3 (1.4)	1†	0	2	0	206	110	91	5
Any PCR+	64	47 (73)	27	5	10	5	17	11	6	0
Total	258‡	48 (19)	28	5	10	5	210	111	94	5

*EIA, enzyme immunoassay; Ig, immunoglobulin; serodiagnoses (SDG), IgM positive and/or IgG conversion or increase; no serodiagnoses, IgM negative and no seroconversion/increase; SC, seroconversion; SI, serologic increase (≥-fold increase in IgG); IgG+, constant level (≤4-fold increase) of IgG in paired serum samples; IgG–, IgG negative in both serum samples; NPA, nasopharyngeal aspirate; Serum+, PCR positive in either or both serum samples.
†One child was IgM negative. The IgG value of the convalescent-phase serum sample barely exceeded the cutoff value and the child was considered as not having an acute human bocavirus infection.
‡One of 259 children (*10*) was not included in this comparison because of depletion of the convalescent-phase serum sample before the PCR (NPA and acute-phase serum samples were PCR negative; both serum samples were IgG positive and IgM negative, indicating immunity).

dren who showed viremia in the first sample (62%) or both samples (87%) had a high (>10^4 copies/mL) HBoV-DNA load in NPAs; most children who showed viremia only in the second sample (58%) had DNA-negative NPAs. These findings suggest a very recent infection. HBoV DNA loads in serum samples ranged from 112 copies/mL to 600,000 copies/mL and did not correlate with NPA DNA loads. Forty-nine (19%) of 258 children had NPAs that were PCR positive, but only 34 (13%) had both NPAs and serum samples that were PCR positive; 194 (75%) children had negative PCR results for both sample types (Table 1). Of long-term follow-up serum samples, only 1/93 was PCR positive; this sample was from a child who was seronegative 5 years earlier and seroconverted during follow-up. All 115 adult serum samples were HBoV PCR negative.

Antibody EIAs

Of 258 wheezing children, 111 (43%) had serologic evidence of past infection; 48 (19%) of acute primary HBoV infection (Table 1; Figure 2). Of the latter group, 32 had detectable IgM with either IgG conversion (27/48) or a diagnostic increase (5/48); a total of 15 had IgM with no IgG (5/48) or with a constant IgG (10/48) absorbance value. A girl 1 year of age showed seroconversion in a convalescent-phase serum sample weakly positive for IgG but she had no IgM in either sample (Table 1). All other children who showed seroconversion or an increase in IgG were IgM positive. Incidence rates of serologically verified, acute, primary HBoV infection among wheezing children varied with age, with a peak of 28% in children 1–<2 years of age (Table 2). Prevalence of HBoV immunity increased with age, reaching 100% at 7 years of age (Table 2). This study included 27 (10%) of 258 children <6 months of age (median 4.8 months, mean 4.7 months). Eight (30%) of 27 children were IgG positive; IgG in 7 of these children was presumably of maternal origin. One infant (3.7%), a 3.6-month-old boy, had a serologically verified acute HBoV infection.

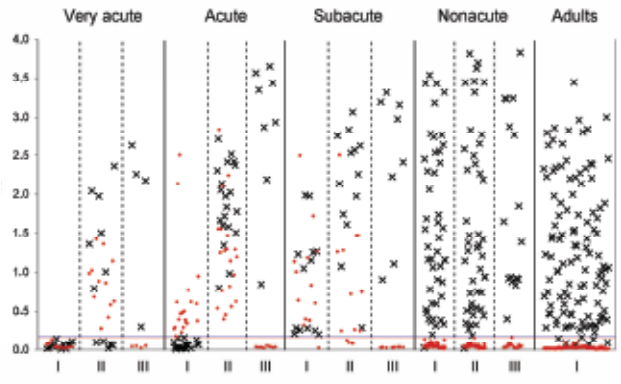

Figure 2. Scatter plots of individual absorbance values at 492 nm (A_{492}) of immunoglobulin (Ig) G (×) and IgM (red dots) against human bocavirus (HBoV) in enzyme immunoassays (EIAs) for acute-phase (I), convalescence-phase (II), and 5-year follow-up (III) serum samples from wheezing children and single serum samples from young healthy adults, Finland. The 45 children with confirmed acute HBoV infections (by viremia and serodiagnosis) were divided into 3 groups according to the degree of acuteness (very acute, acute, and subacute) on the basis of findings in I and II serum samples. Very acute, I sample seronegative but II sample IgM positive (n = 12); acute, I sample IgM positive but IgG showed seroconversion (n = 20); subacute, IgG positive with a diagnostic increase or constant level in I and II samples, IgM positive (n = 13). Also shown are results for children without viremia or serodiagnosis (nonacute [for only the first 45 children with seropositive samples]), and young healthy adults (n = 115). EIA cutoffs are indicated by a blue line (IgG; 0.188) or a red line (IgM; 0.167). Dots below the cutoff lines indicate samples with absorbance values less than the negative cutoff values (i.e., IgM– and IgN– results).

Table 2. Incidence rates of serologically verified acute human bocavirus infections and prevalence rates of bocavirus immunity among 258 wheezing children, Finland*

Characteristic	Age group							
	<6 mo	6–<12 mo	1–<2 y	2–<3 y	3–<4 y	4–<7 y	7–<15 y	Total
Incidence	1/26 (4)	9/37 (24)	25/88 (28)	5/42 (12)	3/25 (12)	2/19 (10)	0/14 (0)	45/251 (18)
Prevalence	1/21† (5)	5/37 (13)	28/88 (32)	30/42 (71)	16/25 (64)	16/19 (84)	14/14 (100)	110/246† (45)

*Values are no. positive/no. tested (%). Discrepant results of enzyme immunoassay and PCR results from 7 children were not included (see Table 1 and text).
†Five infants had maternal antibodies (defined as waning immunoglobulin G absorbance values in paired serum samples). Two other infants became seronegative and were considered virus negative.

Comparison of qPCR and EIA results for 258 children is shown in Table 1. Among 49 children who showed viremia, 45 (92%) had a serodiagnosis of HBoV infection: i.e., had IgM or an increase in IgG. Of the remaining 4 viremic children, 1 was seropositive and 3 were seronegative; 2 of the seronegative children showed viremia in a second sample, possibly indicating a very acute infection (their NPA samples at the time of admission had been PCR negative). Among 209 nonviremic children, 206 (99%) showed nondiagnostic serologic results (Table 1). Only 3 (1.4%) of 209 children had a serodiagnosis: the 1-year-old girl with an apparent false seroconversion described above and 2 children with PCR-positive NPAs and a positive result for IgM but a constant IgG level, which suggests a subacute infection.

Of 49 (19%) of 258 HBoV NPA PCR-positive children, 35 (71%) had a serodiagnosis, 33 of whom were also viremic (Table 1). Of 28 patients with a high load of HBoV DNA in NPAs, 27 (96%) had an HBoV serodiagnosis, compared with only 8 (38%) of 21 with a low DNA load. Conversely, among 209 children without HBoV DNA in NPAs, 13 (6%) had a serodiagnosis, of whom 12 were also viremic.

Among 34 (13%) of 258 children who were HBoV PCR positive by both NPAs and serum samples, 33 (97%) had a serodiagnosis; the remaining child (a girl 6 months of age) was seronegative and viremic only in the second sample, which indicated a very acute infection. In contrast, of 194 (75%) of 258 children who were HBoV PCR negative in both NPAs and serum, only 1 (0.5%) had a serodiagnosis (the IgM-negative 1-year-old girl). Conversely, of 48 (19%) of 258 children who had a serologically diagnosed acute HBoV infection, 35 (73%) were PCR positive for NPAs, 45 (94%) were viremic, and 47 (98%) were PCR positive for NPAs or serum (Table 1). If one considers a positive PCR result for serum as the standard for diagnosis (n = 258), our EIA had a sensitivity of 92%, a specificity of 99%, and a positive predictive value of 94%. If PCR positivity for NPAs and serum is the standard for diagnosis (n = 128), the sensitivity is 97%, the specificity is 99.5%, and the positive predictive value is 97%.

All 258 children had been tested for 16 respiratory viruses (*10*). Of 12 (4.6%) with a serologically diagnosed acute HBoV single infection, 12 were viremic and 10 (83%) were HBoV DNA positive in NPAs (all with high DNA loads). Among 39 HBoV NPA PCR-positive children coinfected with 1 or 2 other respiratory viruses (rhinovirus, enterovirus, RSV, adenovirus, influenza A virus, or parainfluenza virus), 25 (64%) had a serologically diagnosed primary HBoV infection; 17 (94%) of 18 with a high HBoV DNA load and 8 (38%) of 21 with a low HBoV DNA load had primary infections. Among viremic children with serologically confirmed acute HBoV infections, 33 (73%) of 45 had co-infections compared with 12 (92%) of 13 children with HBoV DNA in NPAs but without serodiagnosis or viremia; this difference was not significant (p = 0.26).

Follow-up serum samples obtained 5–8 years later were available for 93 of 258 children. Of 41 IgG-negative children, 38 (93%) had seroconverted, and all 21 acutely infected and all initially IgG-positive children were still IgG positive and had no IgM or HBoV DNA. Of 115 young adults, none had IgM, 110 (96%) had IgG (including 2 with borderline results), and none were viremic. Absorbance values are shown in Figure 2.

Serodiagnostic findings for infection with parvovirus B19 (IgM positive or low epitope-type specificity index) (*18,20*) were not observed among children with a serodiagnosis of HBoV infection. Among other children, 3 were IgM positive for parvovirus B19, of whom 1 was seronegative for HBoV and 2 were seropositive for HBoV.

Clinical Characteristics

The 258 children tested for 16 viruses were analyzed for clinical characteristics. Median age of 46 children with acute HBoV infection diagnosed by serologic analysis and PCR of serum was 1.3 years (range 0.3–6.1 years), median age of 91 of 258 nonexposed seronegative children was 1.1 years (range 0.2–4.2 years), and median age of 110 of 258 children with HBoV immunity was 2.8 years (range 0.5–5.2 years) (p<0.0001).

Clinical data were compared among children infected only with HBoV (n = 12), rhinovirus (n = 56), RSV (n = 36), and HBoV and any other virus (n = 34) (Table 3), HBoV and rhinovirus (n = 14), and HBoV and RSV (n = 7). Among single infections, RSV induced wheezing earliest in life (median 0.8 years), followed by HBoV (1.4 years) and rhinovirus (2.1 years; p<0.0001). Age-adjusted comparison of single infections showed longer duration of hospitalization (p = 0.0069), longer duration of cough (p

Table 3. Clinical characteristics of pediatric patients with acute wheezing caused by HBoV, rhinovirus, RSV, or mixed virus infections*†

	Virus infection				Between groups infected with: Single virus (n = 104)		Between groups infected with: Single + mixed (n = 46)‡
Factor	HBoV (n = 12)	Mixed (n = 34)	Rhinovirus (n = 56)	RSV (n = 36)	p value§	Adjusted p value¶	p value§
Age, y	1.4 (0.8–3.2)	1.3 (0.3–6.1)	2.2 (0.4–12.5)	0.9 (0.3–4.5)	<0.0001	–	0.57
Male, no. (%)	9 (75)	23 (68)	36 (64)	20 (56)	0.45	0.073	0.64
At admission							
Severity of illness, scale 0–12	7 (4–10)	7 (2–10)	6 (2–10)	7 (4–10)	0.057	0.15	0.43
% Oxygen saturation	97 (88–99)	96 (91–99)	96 (88–100)	96 (89–99)	0.98	0.97	0.95
Temperature, °C	37.6 (36.1–39.1)	37.7 (36.2–39.5)	37.4 (36.2–39.3)	37.9 (36.3–40.1)	0.0014	0.032	0.71
CRP, mg/L	7.50 (0–78)	10 (0–45)	18 (0–191)	8 (0–96)	0.25	0.81	0.48
Leukocyte count, × 10^9/L	8.50 (6.3–11.9)	11.1 (5.1–23.6)	12.1 (5.6–20.8)	9.4 (4.9–20.7)	0.0003	<0.0001	0.029
Duration of hospitalization, h	30 (18–78)	27 (6–90)	18 (6–74)	38 (6–138)	<0.0001	0.0066	0.12
Duration of cough, d	15 (4–66)	11 (2–38)	8 (1–36)	11 (4–22)	0.0022	0.020	0.062
Before admission	5 (1–60)	3 (1–28)	2 (0–19)	4 (1–14)	<0.0001	<0.0001	0.038
After hospitalization	6 (2–14)	5 (0–14)	6 (0–14)	4 (0–13)	0.40	0.42	0.36
Moderate–severe after hospitalization	1 (0–8)	1 (0–14)	2 (0–14)	0 (0–4)	0.019	0.0052	0.34
Duration of breathing difficulty, h	4 (1–9)	4 (1–11)	3 (0–36)	6 (1–14)	<0.0001	0.0047	0.48
Before admission	1 (0–7)	1 (0–7)	1 (0–19)	2 (0–6)	0.040	0.67	0.63
After hospitalization	0 (0–4)	1 (0–10)	0 (0–14)	3 (0–11)	0.028	0.071	0.40
Moderate–severe after hospitalization	0 (0–0)	0 (0–6)	0 (0–14)	0 (0–1)	0.39	0.49	0.13
Other symptoms, no. (%) patients							
Acute otitis media	4 (33)	16 (47)	16 (29)	26 (72)	0.0003	0.073	0.41
Nonrespiratory symptoms							
Diarrhea	1 (8)	4 (12)	2 (4)	7 (19)	0.072	0.21	0.74
Balance problems	0	1 (3)	2 (4)	2 (6)	0.92	0.53	0.45
Rash	0	0	2 (4)	0	1.00	1.00	–
Arthritis or arthralgia	0	0	0	0	–	–	–

*Mixed infections consisted of HBoV plus ≥1 virus. Values are median (range) except as indicated. HBoV, human bocavirus; RSV, respiratory syncytial virus; CRP, C-reactive protein.
†Analysis by regression analysis using generalized linear models after logarithmic transformation for continuous data or by logistic regression analysis for categorical data. Significant intergroup differences were found when persons with HBoV infections were compared with those with other infections: age: HBoV vs. RSV, p = 0.036; leukocyte count: HBoV vs. rhinovirus, p = 0.0009 unadjusted and p = 0.0002 adjusted; duration of hospitalization: HBoV vs. rhinovirus, p = 0.0027 unadjusted and p = 0.0069 adjusted; duration of cough: HBoV vs. rhinovirus, p = 0.0009 unadjusted and p = 0.0012 adjusted; duration of cough before admission: HBoV vs. rhinovirus, p<0.0001 unadjusted and p<0.0001 adjusted and HBoV vs. RSV, p = 0.028 unadjusted and p = 0.019 adjusted; acute otitis media: HBoV vs. RSV, p = 0.0005 unadjusted.
‡A child with 1 PCR-positive nasopharygeal aspirate and a PCR-negative serum sample was classified as having a subacute HBoV infection. In Table 1, he was 1 of 3 nonviremic children with a serodiagnosis for HBoV infection.
§Unadjusted.
¶Adjusted for age.

= 0.0012), and longer duration of cough before admission (p<0.0001) for patients with acute HBoV infections than for patients with rhinovirus infections. However, children infected with rhinovirus had a higher leukocyte count at the time of admission (p = 0.0002). When compared with patients infected with RSV, patients infected with HBoV showed longer duration of cough before admission (p = 0.019). Differences in clinical variables were not observed for rhinovirus or RSV, whether in children with single infections or those co-infected with HBoV. We found no differences between children co-infected with HBoV and 1 or 2 other viruses. Nonrespiratory symptoms, including diarrhea, were rare (Table 3).

Acute HBoV infection was found in children with bronchiolitis. Among children <2 years of age, acute HBoV infection was detected in 26 (27%) of 95 children having their first wheezing episode and in 35 (25%) of 141 children having their first or recurrent wheezing episode, excluding asthmatic children (defined as children considered for initiation of daily long-term control therapy according to the recent US guidelines for diagnosis and management of asthma [*21*]). Children with HBoV and RSV single infections showed a similar overall severity of illness (median 7, range 4–10 on a scale of 0–12), whereas acute otitis media (AOM) was more frequent among children with RSV single infections (p = 0.0005) (Table 3).

Discussion

HBoV infections have been commonly diagnosed by PCR of respiratory tract samples. Only a few serologic studies have been reported; these studies have addressed mainly epidemiologic issues (*11–15*). However, Endo et al. documented seroconversions by immunofluorescent analysis in 4 HBoV PCR-positive patients (*12*), and Lindner et al. detected IgM against HBoV by EIA in 12 patients, 10 of whom were viremic children (*15*). In a study of 117 wheezing children, we showed by using immunoblotting and prokaryotically expressed HBoV VP2 capsid antigens that HBoV infections can be diagnosed serologically (*11*). We also showed that the unique region in VP1 is far less immunogenic than the major virus capsid protein VP2. We have now expressed HBoV VP2 VLPs in insect cells for use in IgM and IgG EIAs that are superior to immunoblots in diagnostic performance. We diagnosed acute primary HBoV infections in 48 (19%) of 258 children with expiratory wheezing. Consistent with other reports, no cross-reactivity between 2 human pathogenic parvoviruses (B19 and HBoV), was detected (*11–13,15*). Prevalence rates of immunity increased with age from 5% in infants to >64% in children 2–4 years of age and continued to increase until a maximum of 100% was reached in children 7 years of age. Seroprevalence among young adults was 96%. Furthermore, IgG levels of adults were as diverse as those of children (Figure 2). These results contrast sharply with our previous immunoblot results with denatured VP2 antigen (*11*), which showed decreased seropositivity among children >2 years of age. This difference is likely caused by a time-related conformational dependence of the antibody, similar to immunity to B19 virus (*18,20,22,23*). In other HBoV seroprevalence studies, similar rates were reported (*12,13,15*), which validate the accuracy of our results.

HBoV infection has been shown (by PCR of NPA samples) to be most prevalent in children 6 months to 3 years of age; adults are less affected (*8,24–30*). Consistent with this finding, the incidence of serologically verified acute HBoV infections in our study was highest (28%) during the second year of life; median age of children with acute HBoV infections was 1.3 years. Only 2 children were infected at >4 years of age; 1 of them was seronegative. Children <6 months of age might be protected from infection by maternal antibodies. Rapidly decreasing seroprevalence rates from ≈90% in infants <3 months of age to ≈5% in infants 6 months of age were reported (*12,13*). In our study, 26% of 27 children <6 months of age (including 1 infant <3 months of age) had maternal antibodies, and only 1 child in this age group had an acute HBoV infection.

When we compared serologic results with those of PCR, we found a profound difference between NPA PCR results and serum PCR results. Although results of serodiagnosis were identical to results of serum analysis by PCR, only 71% of NPA-positive children and 6% of NPA-negative children had an HBoV serodiagnosis. However, all but 1 (96%) of the children with a high load of HBoV DNA in NPAs had a serodiagnosis, compared with only 38% of those with a low DNA load. This finding supports the view that a low HBoV DNA load is not evidence of acute primary infection (*10,11,31,32*). Studies of consecutive NPA samples have shown that HBoV DNA can persist in the nasopharynx for several months (*9,33*). We also noted that 22% of children with HBoV DNA in their first serum sample continued to show viremia (with 2 logs less DNA) in the second serum sample obtained an average of 19 days later. That HBoV does not often persist in serum indicates that regardless of its magnitude, viremia is an excellent marker of acute HBoV infection.

The clinical role of codetection of HBoV and other viruses in NPAs has been questioned. It is not easy to determine whether such co-infections are sequential infections or simultaneous viral infections. Serologic analysis is a more precise approach for diagnosis of HBoV infections. When compared with PCR-positive results in serum and NPAs, diagnostic sensitivity and specificity of our antibody EIAs were as high as 97% and 99.5%, respectively; positive predictive value was 97%. We showed by using EIAs that among wheezing children, >60% of co-infections in children with HBoV NPA PCR-positive results, particularly in children with a high HBoV DNA load, are acute primary HBoV infections and should be considered in the diagnosis of respiratory disease. No differences in occurrence of co-infections were observed in children with a serologically confirmed diagnosis of infection with HBoV compared with children positive for HBoV by only PCR of NPAs.

Several groups have compared clinical features of HBoV infections with those of other respiratory virus infections. In those studies, diagnoses of infection with HBoV were based only on PCR positivity of NPAs, which as we showed, is not an ideal marker for detection of acute HBoV infection. We assessed clinical findings of our patients with serologically verified acute HBoV infections. Comparison of HBoV-induced wheezing with that induced by rhinovirus is notable because rhinovirus is commonly associated with wheezing in older children and has been recognized as a risk factor for recurrent wheezing and asthma in young children (*34–36*). Also notable is a comparison of HBoV and RSV because RSV is the dominant cause of bronchiolitis in infants (*37,38*). Our data showed that wheezing induced by RSV occurred at the youngest age (median 10 months), followed by that induced by HBoV (17 months), and rhinovirus (25 months). Age-adjusted comparisons showed that HBoV-infected children were hospitalized longer than rhinovirus-infected children. Illnesses after HBoV infection lasted longer than illnesses after rhinovirus infection. However, we did not demonstrate that co-infection with HBoV would increase

illness duration or severity, as has been reported for rhinovirus- and RSV-induced bronchiolitis (*39*).

Children with HBoV co-infections seemed to have more AOM (47%) than those with single HBoV infections (33%), but this difference could be explained by inclusion of 7 RSV-positive children in this group. The highest rate of AOM (72%) was in children with RSV-induced wheezing. Alper et al. reported differences in frequencies of various respiratory viruses associated with AOM (HBoV was not included), but statistical significance was not achieved (*40*). We found a difference in the frequency of AOM between children with RSV-induced wheezing and those with HBoV-induced wheezing.

Serologically confirmed primary HBoV infections detected in 12 symptomatic children with no signs of other respiratory virus infections (by PCR, culture, antigen detection, or serologic analysis) demonstrate that HBoV is a cause of acute wheezing in young children. Moreover, the fact that acute HBoV infection was detected in 27% of hospitalized children who were <2 years of age when they had their first episode of wheezing indicates that HBoV is a causative agent of bronchiolitis with clinical severity comparable with that of RSV. HBoV respiratory infections can be diagnosed with moderate accuracy by qPCR of NPAs. However, the most reliable methods for diagnosis of acute symptomatic HBoV infection are PCR of serum samples and serologic analysis for IgM and IgG.

Acknowledgments

We thank Kaisu Kaistinen for technical assistance, Päivi Norja for help with the PCR, Hannimari Kallio-Kokko for assistance with the IgM EIA, and Kalle Kantola for preparing the figures.

This study was supported by the Academy of Finland (project 1122539); the Helsinki University Central Hospital Research and Education, and Research and Development funds; the Medical Society of Finland and the Sigrid Jusélius Foundation (M.S.-V., A.L., L.H., K.K., K.H.); the Swedish Research Council; the Swedish Cancer Foundation and the Torsten and Ragnar Söderberg Foundation (T.A.); the Academy of Finland (project 114034); and the Paulo Foundation (T.J.).

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References

1. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005;102:12891–6. DOI: 10.1073/pnas.0504666102
2. Schildgen O, Müller A, Allander T, Mackay IM, Völz S, Kupfer B, et al. Human bocavirus: passenger or pathogen in acute respiratory tract infections? Clin Microbiol Rev. 2008;21:291–304. DOI: 10.1128/CMR.00030-07
3. Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, et al. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000–2005. Clin Infect Dis. 2006;43:585–92. DOI: 10.1086/506350
4. Cilla G, Oñate E, Perez-Yarza E, Montes M, Vicente D, Perez-Trallero E. Viruses in community-acquired pneumonia in children aged less than 3 years old: high rate of viral coinfection. J Med Virol. 2008;80:1843–9. DOI: 10.1002/jmv.21271
5. Fry AM, Lu X, Chittaganpitch M, Peret T, Fischer J, Dowell SF, et al. Human bocavirus: a novel parvovirus epidemiologically associated with pneumonia requiring hospitalization in Thailand. J Infect Dis. 2007;195:1038–45. DOI: 10.1086/512163
6. Kesebir D, Vazquez M, Weibel C, Shapiro ED, Ferguson D, Landry ML, et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis. 2006;194:1276–82. DOI: 10.1086/508213
7. Lahti E, Peltola V, Waris M, Virkki R, Rantakokko-Jalava J, Eerola E, et al. Induced sputum in the diagnosis of childhood community-acquired pneumonia. Thorax. 2009; 64: 252–7.
8. Manning A, Russell V, Eastick K, Leadbetter GH, Hallam N, Templeton K, et al. Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. J Infect Dis. 2006;194:1283–90. DOI: 10.1086/508219
9. von Linstow ML, Høgh M, Høgh B. Clinical and epidemiologic characteristics of human bocavirus in Danish infants: results from a prospective birth cohort study. Pediatr Infect Dis J. 2008;27:897–902. DOI: 10.1097/INF.0b013e3181757b16
10. Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, et al. Human bocavirus and acute wheezing in children. Clin Infect Dis. 2007;44:904–10. DOI: 10.1086/512196
11. Kantola K, Hedman L, Allander T, Jartti T, Lehtinen O, Ruuskanen O, et al. Serodiagnosis of human bocavirus infections. Clin Infect Dis. 2008;46:540–6. DOI: 10.1086/526532
12. Endo R, Ishiguro N, Kikuta H, Teramoto S, Shirkoohi R, Ma X, et al. Seroepidemiology of human bocavirus in Hokkaido Prefecture, Japan. J Clin Microbiol. 2007;45:3218–23. DOI: 10.1128/JCM.02140-06
13. Kahn JS, Kesebir D, Cotmore S, D'Abramo A Jr, Cosby C, Weibel C, et al. Seroepidemiology of human bocavirus defined using recombinant virus-like particles. J Infect Dis. 2008;198:41–50. DOI: 10.1086/588674
14. Lin F, Guan W, Cheng F, Yang N, Pintel D, Qiu J. ELISAs using human bocavirus VP2 virus-like particles for detection of antibodies against HBoV. J Virol Methods. 2008;149:110–7. DOI: 10.1016/j.jviromet.2007.12.016
15. Lindner J, Karalar L, Zehentmeier S, Plentz AS, Pfister H, Struff W, et al. Humoral immune response against human bocavirus VP2 virus-like particles. Viral Immunol. 2008;21:443–9. DOI: 10.1089/vim.2008.0045
16. Jartti T, Lehtinen P, Vuorinen T, Osterback R, van den Hoogen B, Osterhaus AD, et al. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. Emerg Infect Dis. 2004;10:1095–101.
17. Ekman A, Hokynar K, Kakkola L, Kantola K, Hedman L, Bondén H, et al. Biological and immunological relations of human parvovirus B19 genotypes 1–3. J Virol. 2007;81:6927–35. DOI: 10.1128/JVI.02713-06
18. Kaikkonen L, Lankinen H, Harjunpää I, Hokynar K, Söderlund-Venetmo M, Oker-Blom C, et al. Acute-phase-specific heptapeptide epitope for diagnosis of parvovirus B19 infection. J Clin Microbiol. 1999;37:3952–6.

19. Kallio-Kokko H, Vapalahti O, Lundkvist Å, Vaheri A. Evaluation of Puumala virus IgG and IgM enzyme immunoassays based on recombinant baculovirus-expressed nucleocapsid protein for early nephropathia epidemica diagnosis. Clin Diagn Virol. 1998;10:83–90. DOI: 10.1016/S0928-0197(97)10019-8
20. Söderlund M, Brown CS, Spaan WJ, Hedman L, Hedman K. Epitope type-specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis. 1995;172:1431–6.
21. National Asthma Education and Prevention Program. Expert Panel Report 3. Guidelines for the diagnosis and management of asthma. Bethesda (MD): National Heart, Lung, and Blood Institute; 2007.
22. Corcoran A, Mahon BP, Doyle S. B cell memory is directed toward conformational epitopes of parvovirus B19 capsid proteins and the unique region of VP1. J Infect Dis. 2004;189:1873–80. DOI: 10.1086/382963
23. Enders M, Schalasta G, Baisch C, Weidner A, Pukkila L, Kaikkonen L, et al. Human parvovirus B19 infection during pregnancy: value of modern molecular and serological diagnostics. J Clin Virol. 2006;35:400–6. DOI: 10.1016/j.jcv.2005.11.002
24. Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol. 2006;78:1232–40. DOI: 10.1002/jmv.20689
25. Arnold JC, Singh KK, Spector SA, Sawyer MH. Human bocavirus: prevalence and clinical spectrum at a children's hospital. Clin Infect Dis. 2006;43:283–8. DOI: 10.1086/505399
26. Ma X, Endo R, Ishiguro N, Ebihara T, Ishiko H, Ariga T, et al. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol. 2006;44:1132–4. DOI: 10.1128/JCM.44.3.1132-1134.2006
27. Qu XW, Duan ZJ, Qi ZY, Xie ZP, Gao HC, Liu WP, et al. Human bocavirus infection, People's Republic of China. Emerg Infect Dis. 2007;13:165–8.
28. Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, Mackay IM. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol. 2006;35:99–102. DOI: 10.1016/j.jcv.2005.09.008
29. Smuts H, Hardie D. Human bocavirus in hospitalized children, South Africa. Emerg Infect Dis. 2006;12:1457–8.
30. Weissbrich B, Neske F, Schubert J, Tollmann F, Blath K, Blessing K, et al. Frequent detection of bocavirus DNA in German children with respiratory tract infections. BMC Infect Dis. 2006;6:109. DOI: 10.1186/1471-2334-6-109
31. Gerna G, Piralla A, Campanini G, Marchi A, Stronati M, Rovida F. The human bocavirus role in acute respiratory tract infections of pediatric patients as defined by viral load quantification. New Microbiol. 2007;30:383–92.
32. Jacques J, Moret H, Renois F, Léveque N, Motte J, Andréoletti L. Human bocavirus quantitative DNA detection in French children hospitalized for acute bronchiolitis. J Clin Virol. 2008;43:142–7. DOI: 10.1016/j.jcv.2008.05.010
33. Brieu N, Guyon G, Rodiére M, Segondy M, Foulongne V. Human bocavirus infection in children with respiratory tract disease. Pediatr Infect Dis J. 2008;27:969–73. DOI: 10.1097/INF.0b013e31817acfaa
34. Jackson DJ, Gangnon RE, Evans MD, Roberg KA, Anderson EL, Pappas TE, et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am J Respir Crit Care Med. 2008;178:667–72. DOI: 10.1164/rccm.200802-309OC
35. Kotaniemi-Syrjänen A, Vainionpää R, Reijonen T, Waris M, Korhonen K, Korppi M. Rhinovirus-induced wheezing in infancy: the first sign of childhood asthma? J Allergy Clin Immunol. 2003;111:66–71. DOI: 10.1067/mai.2003.33
36. Lehtinen P, Ruohola A, Vanto T, Vuorinen T, Ruuskanen O, Jartti T. Prednisolone reduces recurrent wheezing after a first wheezing episode associated with rhinovirus infection or eczema. J Allergy Clin Immunol. 2007;119:570–5. DOI: 10.1016/j.jaci.2006.11.003
37. Papadopoulos NG, Tsolia M, Bossios A, Astra E, Prezerakou A, Gourgiotis D, et al. Association of rhinovirus infection with increased disease severity in acute bronchiolitis. Am J Respir Crit Care Med. 2002;165:1285–9. DOI: 10.1164/rccm.200112-118BC
38. Rakes GP, Arruda E, Ingram J, Hoover GE, Zambrano JC, Hayden FG, et al. Rhinovirus and respiratory syncytial virus in wheezing children requiring emergency care. IgE and eosinophil analyses. Am J Respir Crit Care Med. 1999;159:785–90.
39. Richard N, Komurian-Pradel F, Javouhey E, Perret M, Rajoharison A, Bagnaud A, et al. The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis. Pediatr Infect Dis J. 2008;27:213–7. DOI: 10.1097/INF.0b013e31815b4935
40. Alper CM, Winther B, Mandel E, Hendley J, Doyle W. Rate of concurrent otitis media in upper respiratory tract infections with specific viruses. Arch Otolaryngol Head Neck Surg. 2009;135:17–21. DOI: 10.1001/archotol.135.1.17

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Recent Ancestry of Kyasanur Forest Disease Virus

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Kyasanur Forest disease virus (KFDV) is enzootic to India and maintained in ticks, mammals, and birds. It causes severe febrile illness in humans and was first recognized in 1957 associated with a high number of deaths among monkeys in Kyasanur Forest. Genetic analysis of 48 viruses isolated in India during 1957–2006 showed low diversity (1.2%). Bayesian coalescence analysis of these sequences and those of KFDVs from Saudi Arabia and the People's Republic of China estimated that KFDVs have evolved at a mean rate of ≈6.4 × 10^{-4} substitutions/site/year, which is similar to rates estimated for mosquito-borne flaviviruses. KFDVs were estimated to have shared a common ancestor in ≈1942, fifteen years before identification of the disease in India. These data are consistent with the view that KFD represented a newly emerged disease when first recognized. Recent common ancestry of KFDVs from India and Saudi Arabia, despite their large geographic separation, indicates long-range movement of virus, possibly by birds.

Kyasanur Forest disease virus (KFDV) is a member of the mammalian tick-borne virus group (previously referred to as the tick-borne encephalitis serogroup) of the family *Flaviviridae* and genus *Flavivirus* (*1*). In addition to KFDV, this group contains Louping ill, tick-borne encephalitis, Omsk hemorrhagic fever, Langat, Powassan, Royal Farm, and Gadgets Gully viruses. KFD was first recognized in 1957 in the Kyasanur Forest of Shimoga District, Karnataka State, India, when a disease causing a high number of deaths was observed in 2 species of monkeys: the black-faced langur (*Semnopithecus entellus*, earlier known as *Presbytis entellus*) and the red-faced bonnet monkey (*Macaca radiata*).

Human cases were also found among persons who visited forests to collect firewood, grass, and other forest products. Human disease is characterized by an incubation period of ≈3–8 days, followed by chills, frontal headache, body ache, and high fever for 5–12 days, and a case-fatality rate ≥30% (*2*). During infection by KFDV, virus titer remains high ≤10 days after onset of symptoms, as reported by Bhat et al. (*3*). However, Upadhyaya et al. (*4*) found that viremia in patients lasted for 12–13 days of illness and unlike most other flaviviruses, remains high during the first 3–6 days with titers as high as 3.1 × 10^6 PFU/mL.

Continuing deaths in monkeys and an average of 400–500 human cases have been seen annually over the past 5 decades, commonly occurring in evergreen, semi-evergreen, and neighboring, moist, deciduous forest areas. An array of tick species, mainly *Haemaphysalis spinigera*, act as vectors for KFDV (*5*). This species of tick is widely distributed in tropical evergreen and deciduous forests of southern and central India and Sri Lanka. KFDV has also been isolated from 7 other species of this genus and from *Dermacentor* and *Ixodes* ticks. This disease is transmitted by ticks among ground birds and small mammals such as the white-tailed rat, white-bellied rat, shrew, and bat. High titers of virus can be obtained after experimental infection of black-napped hares, porcupines, flying squirrels, Malabar giant squirrels, three-striped squirrels, gerbils, mice, long-tailed tree mice, and shrews (*2–9*).

Until 1971, KFDV was endemic to the Sagar, Sorab, and Shikaripur taluks (counties) of Shimoga District (Figure 1). By 1972, a new focus of virus activity appeared in Sirsi Taluk, Uttara Kannada District. Many KFDV isolates were obtained from Karnataka during 1957–1972 and maintained in a repository at the National Institute of Virology

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DOI: 10.3201/eid1509.080759

(NIV) in Pune, India. However, the virus was found to be highly infectious, as shown by numerous infections in field and laboratory personnel (*2*,*10*), which resulted in suspension of work with this virus until an appropriate BioSafety Level-3 laboratory was built at NIV in 2004. In 2006, this laboratory isolated a virus from a serum sample of a patient suspected of having KFD that was obtained from the Virus Diagnostic Laboratory in Shimoga.

More recent studies have identified KFDV in Saudi Arabia and the People's Republic of China (*11*,*12*). During 1994–1995, a virus was isolated from hemorrhagic fever patients in the Makkah region of Saudi Arabia and identified as a KFDV variant, referred to as the Alkhurma variant or subgroup (*11*,*13*,*14*). The prototype strain of KFDV from Saudi Arabia (strain 1176, isolated in 1995) and the KFDV reference strain from India (P-9605, isolated in 1957) differ from each another by only 8% at the genome nucleotide level, despite their temporal (38 years) and geographic (≈4,000 km) separation. A virus initially referred to as Nanjianyin virus, isolated in 1989 from a febrile patient in Nanjian County in the Hengduan Mountain region of Yunnan Province in southwestern China, was recently identified as a strain of KFDV (*12*). However, it is unclear whether this KFDV 1989 isolate from China is an authentic virus isolate because it is virtually identical at the nucleotide level with the 1957 reference strain from India (P-9605), despite their being isolated 32 years and almost 3,000 km apart. The P-9605 strain was distributed widely to arbovirus reference laboratories. Reference KFDV virus was used as part of the analysis of serum samples from Yunnan Province (*15*,*16*).

Results of molecular epidemiologic studies have suggested that tick-borne flaviviruses have evolved slowly while dispersing north and west across Asian and European forests during the past few millennia (*17*–*19*). This pattern is different from that of rapidly evolving mosquito-borne flaviviruses, many of which can be transported long distances by migratory birds, persons, animals, or mosquito eggs (*19*,*20*). We examined the diversity and evolution of KFDV and present data that indicated that KFDV isolates from India, Saudi Arabia, and China share a recent common ancestor, indicating long-range movement of this tick-borne flavivirus. In addition, we also estimated the evolution rate of KFDV and compared it with that of mosquito-borne flaviviruses.

Methods

Virus Selection and Reverse Transcription–PCR

Forty-seven representative KFDV isolates from India were chosen for analysis; these isolates were obtained during 1957–1972 (Table 1). Isolates represented viruses from various host species and different geographic locations in Shimoga, Uttara Kannada, and Dakshina Kannada districts, Karnataka State. One KFDV from India isolated in 2006 was also included. Lyophilized KFDV stocks were obtained from the virus repository at the NIV, India, and grown in Vero E6 cell lines. Primers for PCR and phylogenetic analysis were designed to target regions of structural genes (premembrane/envelope) and the nonstructural protein 5 (NS5) gene (viral polymerase) (Table 2).

Total RNA was extracted from 250 μL of infected Vero cell lysates by using Trizol reagent (GIBCO-BRL, Gaithersburg, MD, USA) per the manufacturer's protocol. RNA was dissolved in 50 μL of nuclease-free water. cDNA was prepared separately for structural genes and NS5 by using avian Moloney virus reverse transcriptase (Promega, Madison, WI, USA). Briefly, 10 pM of each gene-specific reverse primer (ER2 and R4 were used for each set of the reverse transcription reactions, respectively) and incubated at 42°C for 45 min and then 85°C for 5 min.

cDNA was amplified by using 1U of Taq DNA Polymerase, 10× PCR buffer (Invitrogen. Carslbad, CA, USA), 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 10.0 pM of each primer pair as described in Table 2 in a reaction volume of 25 μL. PCR conditions included denaturation at 94°C for 5 min; 35 cycles of 1-min steps at 94°C, 55°C, and 72°C; and a 5-min extension at 72°C. Amplified products were analyzed by agarose gel electrophoresis. Bands of interest

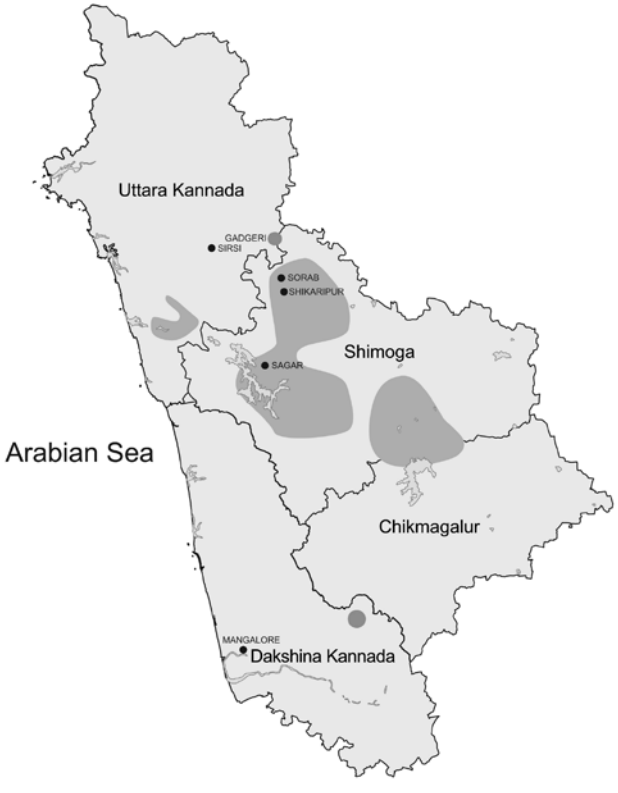

Figure 1. Areas of Karnataka State, India, known to be affected by Kyasanur Forest disease (dark gray shading).

were recovered by using a DNA Gel Extraction Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's protocol. Direct sequencing of the amplified product was conducted by using an ABI 3100 automated DNA sequencer and Big Dye terminator kit (Applied Biosystems, Foster City, CA, USA).

Virus Sequence Analysis

The quality of each sequence was monitored by using Sequence Analysis software version 5.1 (Applied Biosystems). Sequences were assembled by using Kodon software version 2.1 (Appled Maths, Austin, TX, USA). Sequences were processed to give 720 nt of the structural gene (nt positions 500–1220) and 620 nt of the NS5 gene (nt po-

Table 1. Isolates of Kyasanur Forest disease virus analyzed, India*

ID no.	Isolate	Year	Location	Original source	Common name of source
1	W379	1957	Baragi	*Semnopithecus entellus*	Black-faced langur
2	P9605	1957	Shigga	*Homo sapiens*	Human
3	G11333	1957	Barasi	*Haemaphysalis spinigera*	Tick
4	P16011	1958	Kaisodi	*H. sapiens*	Human
5	W3399	1958	Hessare	*S. entellus*	Black-faced langur
6	W6043	1959	Belisiri	*S. entellus*	Black-faced langur
7	W6178	1959	Koppalgadde	*S. entellus*	Black-faced langur
8	G27667	1959	Kunvahalli	*Haemaphysalis spinigera* from dead monkey	Tick
9	P20924	1959	Mullukere	*H. sapiens*	Human
10	P21092	1959	Hadapsar	*H. sapiens*	Human
11	601203	1960	Tudikoppa	*H. sapiens*	Human
12	611661	1961	Sagar Station	*Haemaphysalis turturis*	Tick
13	612057	1961	Barur	*Rattus rattus wroughtoni*	White-bellied rat
14	62844	1962	Hillemarur	*H. spinigera*	Ticks
15	62849	1962	Hillemarur	*R. rattus wroughtoni*	White-bellied rat
16	62957	1962	Hillemarur	*H. sapiens*	Human
17	623969–2	1962	VRC Poona	*H. sapiens*	Human
18	63661	1963	Malvei	*H. sapiens*	Human
19	63696	1963	Suranagadde	*S. entellus*	Black-faced langur
20	64244	1964	Balagodu	*Ixodes petauristae*	Tick
21	64350	1964	Marasa	*Haemaphysalis formosensis*	Tick
22	642034	1964	Kangodu	*H. turturis*	Tick
23	642046	1964	Kangodu	*Haemaphysalis papuana kinneari*	Tick
24	652	1965	Kangodu	*Haemaphysalis wellingtoni*	Tick
25	651521	1965	VRC Poona	*H. sapiens*	Human
26	652980	1965	Vadnala	*Haemaphysalis spp.*	Tick
27	6616	1966	Yelagalale	*S. entellus*	Black-faced langur
28	66364–1	1966	VRC staff, Sagar	2-day acute-phase serum sample, *H. sapiens*	Human
29	66928–2	1966	Sagar	*H. sapiens*	Human
30	664518	1966	Kondagalale	*H. turturis*	Tick
31	67965	1967	Sagar	*H. sapiens*	Human
32	671004	1967	Bhadrapura	*S. entellus*	Black-faced langur
33	673514	1967	Siravala	*H. papuana kinneari*	Tick
34	68142	1968	Holagalale	*S. entellus*	Black-faced langur
35	68159	1968	Siravala	*H. turturis*	Tick
36	68484	1968	Halagalale	*Rattus blanfordi*	White-tailed wood rat
37	681960	1968	Barur	*H. sapiens*	Human
38	692156	1969	Chikkanallur	*H. spinigera*	Tick
39	692163	1969	Thonagodu	*H. sapiens*	Human
40	712419	1971	Nodahalli	*H. spinigera*	Tick
41	716810	1971	Gunjnur	*H. spinigera*	Tick
42	72166	1972	Gadgeri-sirsi	*Haemaphysalis kyasanurensis*	Tick
43	72827	1972	Holekoppa	*S. entellus*	Black-faced langur
44	A106	2006	Chikkanallur	*H. sapiens*	Human
45	W6204	1959	Kannahalli	*S. entellus*	Black-faced langur
46	G27678	1959	Kopalgadde	*H. spinigera*	Tick
47	W1930	1958	Chimnoor	*S. entellus*	Black-faced langur
48	601011	1960	Chikkasakuna	*H. sapiens*	Human

*ID, identification; VRC, Virus Research Centre.

Table 2. Primers used for diagnostic nested reverse transcription–PCR and genotyping of KFD virus, India*

Gene	Primer	Genome location	Primer sequence (5′ → 3′)	Product, bp	Type
preM–env	KFD-EF2	459–478	TGGTGTTCTCTGCGACAGTT	780	Genotyping
	KFD-ER2	1258–1238	TCTGTCACTCTGGTCTCGCTT		
	KFD-EF3†	606–628	TCATTCGAGTGTGTGTCACCATT		
	KFD-ER1†	701–678	TTCCGTATTCCAGTGACACTCGCT		
NS5	KFD-F3	9422–9441	GGCTGAGTCATGGACATCAT	642	
	KFD-R4	11046–11063	TCCACTCGTGTGGATGCT		
	KFD-F4†	9660–9680	TGAGACCTTCTGACGACCGCT		
	KFD-R3†	9801–9819	TCCTTCATCGTCAACTCAT		

*preM, premembrane; env, envelope; KFD, Kyasanur Forest disease; NS5, nonstructural protein 5.
†Internal primers for sequencing some isolates.

sitions 9440–10080) and submitted to the National Center for Biotechnology Information (Bethesda, MD, USA) NCBI (GenBank accession nos. EU293242–EU293289 and EU293290–EU293337, respectively). Multiple sequence alignments were generated by using the MAFFT function (*21*) in SeaView (*22*). Nucleotide and amino acid proportional distances were calculated and compared for each virus with their respective date of isolation.

A partition homogeneity test (*23*) was conducted by using PAUP* 4.0b10 (*24*) to demonstrate that it was not inappropriate to analyze the 720-nt structural gene fragment and 620-nt NS5 gene fragment as a colinearized or concatenated single sequence. Phylogenetic analysis was performed on the colinearized sequence from each of the 48 KFDV isolates from India (Table 1) along with the corresponding gene regions available in GenBank for additional KFDVs: a1989 KFDV isolate (Nanjianyin) reportedly from China (EU918174, NS5 and EU918175, polyprotein) and 2 KFDVs from Saudi Arabia isolated in 1995 (AF331718) and 2004 (DQ154114).

The Modeltest 3.7 software program (*25*) was used to examine 56 models of nucleotide substitution to determine the model most appropriate for Bayesian coalescent analysis of the KFDV dataset. The general time reversible evolutionary model incorporating invariant sites (GTR + I) was found to be the best fit to the data according to the Akaike information criterion. Bayesian phylogenetic analysis was conducted by using BEAST, BEAUTi, and Tracer analysis software (*26*) with the GTR + I model. Preliminary analyses were run for 10,000,000 generations to select the clock and demographic models most appropriate for the KFDV dataset. An analysis of the marginal likelihoods indicated that the relaxed lognormal molecular clock and constant population size model was decisively chosen ($\log_{10}$ Bayes factors of 3.113) for the KFDV dataset. Final data analysis included a Markov chain Monte Carlo chain length of 50,000,000 generations with sampling every 1,000 states.

Results

Comparison of nucleotide sequences of colinearized fragments of structural (720 nt) and NS5 (620 nt) genes of 48 KFDV isolates from India collected over the past 5 decades (Table 1) showed a low level of diversity among these viruses (GenBank accession nos. EU29242–EU29337). A maximum of 1.2% nt and 0.5% aa differences were seen among these viruses; the most divergent virus was the A106 virus isolated in 2006. Most viruses were isolated during 1957–1972. That the 2006 virus isolate is the most divergent is consistent with the 34-year gap in sampling. As expected, little diversity was seen among the virus isolates irrespective of the host, which included humans, black-faced langurs, red-faced bonnet monkeys, various tick species (*H. spinigera*, *H. kyasanurensis*, *H. turturis*, *H. papuana kinneari*, *H. wellingtoni*, *H. formosensis*, and *Ixodes petauristae*), and rodents (*Rattus rattus wroghtoni* and *R. blanfordi*) (Table 1). The sequence of the 1957 KFDV reference strain (P9605) from India and strain 651521 isolated from an NIV laboratory staff member in 1965 were identical, despite their 8-year separation. However, the staff member was accidently infected while handling reference KFDV, which provided an explanation for this anomaly.

All KFDV isolates from India differed from the Alkhurma variant of KFDV (*27*) found in Saudi Arabia by ≈8%–9% at the nucleotide level. This finding is similar to the extent of diversity (8%) reported in a comparison of the complete genome of a KFDV isolate from India with that of an isolate from Saudi Arabia (*28*). In contrast, the 1989 KFDV isolate (Nanjianyin) reportedly from China (*12*), differed by only 1 nt (1/1,320 [0.08%]) from the 1957 KFDV reference strain (P9605) from India and the laboratory infection strain 651521. It is notable that of the 48 KFDV strains from India analyzed, the KFDV strain from China should be most similar to strain P9605, a reference strain that was distributed worldwide to arbovirus reference laboratories. The KFDV 1989 isolate from China is virtually identical at the nucleotide level to the 1957 reference strain (P9605) from India, despite their being isolated 32 years and almost 3,000 km apart, which suggests that the strain from China is not an authentic virus isolate. A reference KFDV from India appears to have been used in the analysis of serum samples from Yunnan Province (*15*,*16*), which suggests a potential source of laboratory cross-contamination. In addition, the 2 sequence fragments

(EU918174 for NS5 and EU918175 for the polyprotein) of the KFDV isolate reportedly from China appear to contain several sequence analysis errors; neither fragment encodes a functional protein because of creation of a stop codon and 2 frame shifts relative to KFDV reference sequences (AY323490 and EU480689).

Bayesian coalescent analysis of sequence differences among the 48 KFDV isolates from India (1957–2006), the isolates from Saudi Arabia (1995–2004) (*28*,*29*), and the reported isolate from China (1989) (*12*) was conducted to estimate the rate of evolution and time to the most recent common ancestor (MRCA) for these viruses (Figure 2). These viruses were estimated to be evolving at a mean rate of 6.4×10^{-4} substitutions/site/year (95% highest probable density [HPD] $4.1–8.8 \times 10^{-4}$ substitutions/site/year). This estimate is similar to rates for other flaviviruses analyzed by using similar Bayesian coalescent methods, including a rate of 2.17×10^{-4} substitutions/site/year obtained for 23 St. Louis encephalitis viruses collected during 1933–2001 (*30*) and a rate of 4.2×10^{-4} substitutions/site/year for yellow fever virus (*31*). The finding of similar evolutionary rates for tick-borne and mosquito-borne flaviviruses was unexpected, given earlier assertions that evolution of tick-borne viruses was more gradual than rapidly evolving mosquito-borne viruses (*19*).

Analysis estimated that the mean time to the MRCA for all the KFDV isolates was only 64 years (95% HPD 51–84 years) before 2006 (the year when the most contemporary virus was isolated). The analysis estimated that these viruses shared a common ancestor as recently as ≈1942. Analysis of only KFDV isolates from India provides a slightly more recent estimate of their MRCA (≈1948), just 9 years before identification of the disease in Kyasanur Forest in 1957. This finding correlates well with the perception of local villagers and healthcare providers in the Kyasanur Forest area that this was a newly emerged disease (*32*). Massive deaths of monkeys or compatible human disease in the region were not reported before the 1957 disease outbreak. In the initial years, disease activity was reported in a limited area of ≈100 km² in Sagar and Sorab taluks of Shimoga District. However, after 1972, epizootics and epidemics were recognized in several new foci, increasingly more distant from the original focus.

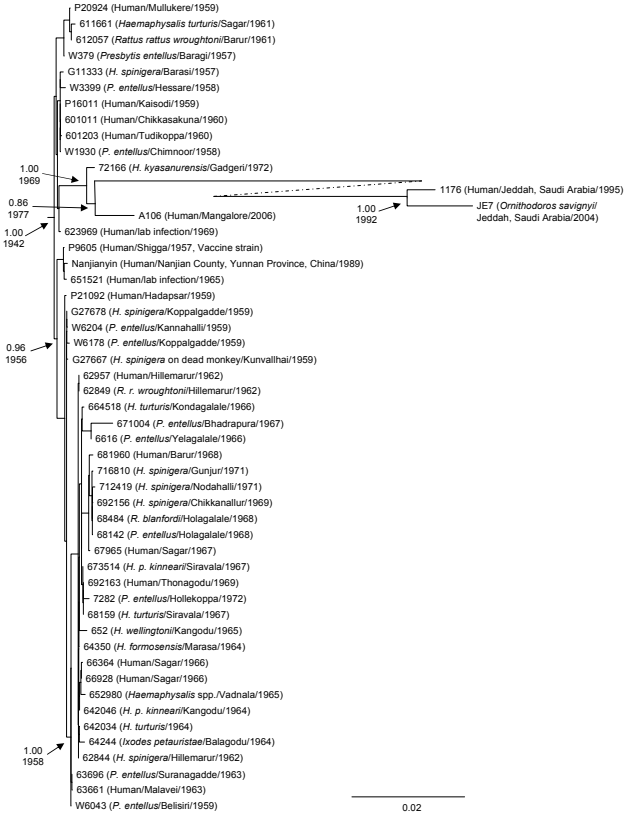

Figure 2. Bayesian coalescent analysis of sequence differences of Kyasanur Forest disease virus isolates from India (1957–2006), People's Republic of China (1989), and Saudi Arabia (1995–2004). Analysis was conducted by using the general time reversible model incorporating invariant sites, a relaxed molecular clock, constant population size, and the BEAST, BEAUTi, and Tracer analysis software (*26*). The maximum clade credibility tree is depicted. Posterior probability values are indicated for clades of interest with the time to most recent common ancestor shown below. Scale bar indicates nucleotide substitutions per site.

Discussion

Most viruses analyzed were isolated in various small hamlets from migrating persons within the early enzootic zone in the Shimoga District (until 1972). Attempts to examine the relationship between genetic differences in a virus isolate relative to geographic location did not show any notable findings because of small differences and distances involved. However, the 1972 virus 72166 was isolated from a tick in the village of Gadgeri in Sirsi (Uttara Kannada District), which is north of Shimoga District. The 2006 virus A106 was isolated from a person south of Shimoga District, in Mangalore (Dakshina Kannada District) and further from the original virus epicenter (Figure 1). Although much of the topology of the virus phylogenetic tree generated by Bayesian coalescent analysis is poorly supported (nodal support posterior probability values <95), there is support for a branch that contains the 72166 1972 and the A106 2006 virus isolates (Figure 2). These data suggest that there may be an association between virus genetic divergence and temporal and geographic spread of KFDV in Karnataka, consistent with the concept of virus spreading over time from an initial focus of activity. Why this initial focus of virus activity occurred in this location and at this time remains unclear, but speculation includes emergence of the virus from a cryptic forest cycle caused by

changes in land use or introduction of the virus from elsewhere by birds. A more complete picture should emerge with analysis of additional virus samples (particularly from the post-1973 period) and complete virus genomes.

Bayesian analysis estimates that the 1995 and 2004 KFDV isolates from Saudi Arabia shared a common ancestor in 1992. The node connecting these viruses with the 2006 KFDV isolate from India was in 1977, and a strongly supported node (1.0) shows that the 1972 and 2006 KFDV isolates from India shared a common ancestor with the viruses from Saudi Arabia in ≈1969. The simplest interpretation of these data and the epidemiologic observations would be that KFDV was introduced from India into Saudi Arabia in the late 1970s or the 1980s.

Similar findings of low genetic diversity and recent common ancestry were reported for the KFDVs from Saudi Arabia in a more limited study of 11 virus isolates collected over a 5-year period (1994–1999). Only 0.4%, 0.6%, and 0.9% genetic diversity were found in the E, NS3, and NS5 gene fragments of these isolates, respectively (*27*). Using these gene fragments along with those of the complete genome sequence of KFDV from India, the authors estimated divergence time by using an older method based on distance analysis of nonsynonymous substitutions. This estimate indicated recent ancestry of these viruses. The KFDV strains from Saudi Arabia were estimated to have diverged from one another over a 4–72 year period and the KFDVs from India and Saudi Arabia were estimated to have diverged 66–177 years ago (*27*).

It is unclear what factors influenced the apparent emergence of KFDV in Shimoga District, India, in 1957 and in the Makkah/Jeddah region in Saudi Arabia in 1994. Also unknown is how this tick-borne virus moved over the large distance between these regions. A considerable amount of knowledge has been accumulated with regard to the ecology of KFDV in India (*32*). The natural history of the virus is complex and involves dynamic cycles of various life stages of Ixodid ticks (primarily *H. spinigera*, but also other *Haemaphysalis* spp. ticks and *Ixodes* ticks) and amplifying (vertebrate) hosts, including rodents and shrews, and possibly monkeys and cattle. Increased human populations in the Sagar and Sorab taluks in the early 1950s may have been the primary catalyst for emergence of KFD in 1957. During 1951–1961, the population of Sagar Taluk increased 116%, bringing with it increases in deforestation, cattle grazing, and extension of paddy fields and cleared grazing areas deeper into previously forested areas (*32*). Expansion of the cattle population may have been a crucial factor because cattle harbor adult forms of *H. spinigera* ticks, and an association between cattle and increases in tick larval density has been described (*32*). Cattle also carry all life stages of other *Haemaphysalis* spp. ticks, which have been shown to be infected with KFDV. Thus, cattle would likely increase tick densities in cleared forest areas most frequented by humans. In addition, rats, shrews, and mice are highly susceptible to KFDV infection, and numerous virus isolates have been obtained from organs of infected animals (*33*). Changes in land use and population densities may have resulted in emergence of KFDV from a cryptic enzootic cycle in this previously heavily forested area.

A high percentage of birds in the affected area are positive for antibodies reactive with KFDV and infested with *Haemaphysalis* spp. and other tick genera, particularly larvae and nymphs (*32*). It is unclear whether birds play a role in the complex virus maintenance cycle in an enzootic zone, but birds carrying virus-infected ticks or migration of viremic birds could spread KFDV over large distances such as those separating areas of KFDV activity in India and Saudi Arabia (*19*,*34*). There is serologic evidence of KFDV, or a related flavivirus in the mammalian tick-borne virus group, in Saurashtra, Gujarat State, on the coast of India on the Arabian Sea and in birds captured outside Karnataka State (*2*,*10*,*32*).

The current known distribution of KFDV is limited to relatively restricted areas of India and Saudi Arabia. However, it is likely that the virus exists in other areas in cryptic enzootic cycles or is associated with unrecognized or undiagnosed disease. This finding, together with the distance separating the KFDV-affected areas in India and Saudi Arabia, despite their relatively recent common ancestry, suggests that KFD has the potential to flare up in other regions because of virus movement or ecologic changes in the area. Clinicians should consider KFD in a differential diagnosis when considering acute febrile cases with compatible symptoms in other regions of Asia and the Middle East.

Acknowledgments

We thank the staff at the virus repository, National Institute of Virology, Pune, India, for providing lyophilized virus stocks; the staff of the Virus Diagnostic Laboratory, Shimoga, India, for providing serum samples; the Council of Scientific and Industrial Research, India, for a senior research fellowship; and Craig Manning for creating the map.

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References

1. Thiel H-J, Collett MS, Gould EA, Heinz FX, Houghton M, Meyers G, et al. *Flaviviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desslberger U, Ball LA, editors. Virus taxonomy: Eighth report of the International Committee on Taxonomy of Viruses. San Diego (CA): Elsevier Academic Press; 2005. p. 981–98.

2. Banerjee K. Kyasanur Forest disease. In: Monath TP, editor. Arboviruses: epidemiology and ecology. Boca Raton (FL): CRC Press; 1990. p. 93–116.
3. Bhatt PN, Work TH, Varma MGR, Trapido H, Narasimha Murthy DP, Rodrigues FM. Isolation of Kyasanur Forest disease from infected humans and monkeys of Shimoga District, Mysore State. Indian J Med Sci. 1966;20:316–20.
4. Upadhyaya S, Narasimha Murthy DP, Anderson CR. Kyasanur forest disease in the human population of Shimoga district, Mysore state (1959–1966). Indian J Med Res. 1975;63:1556–63.
5. Sreenivasan MA, Bhat HR, Rajagopalan PK. The epizootics of Kyasanur Forest disease in wild monkeys during 1964 to 1973. Trans R Soc Trop Med Hyg. 1986;80:810–4. DOI: 10.1016/0035-9203(86)90390-1
6. Bhat UK, Goverdhan MK. Transmission of Kyasanur Forest disease virus by the soft tick, *Ornithodoros cross*. Acta Virol. 1973;17:337–42.
7. Bhat HR, Sreenivasan MA, Nayak SV. Susceptibility of common giant flying squirrel to experimental infection with KFD virus. Indian J Med Res. 1979;69:697–700.
8. Goverdhan MK, Anderson CR. Reaction of *Rattus rattus wroughtoni* to Kyasanur forest disease virus. Indian J Med Res. 1978;67:5–10.
9. Goverdhan MK, Anderson CR. The reaction of *Funambulus tristriatus*, *Rattus blanfordi* and *Suncus murinus* to Kyasanur forest disease virus. Indian J Med Res. 1981;74:141–6.
10. Banerjee K, Bhat HR. Kyasanur forest disease. In: Mishra A, Polasa H, editors. Virus ecology. New Delhi (India): South Asian Publisher; 1984. p. 123–38.
11. Zaki AM. Isolation of a flavivirus related to the tick-borne encephalitis complex from human cases in Saudi Arabia. Trans R Soc Trop Med Hyg. 1997;91:179–81. DOI: 10.1016/S0035-9203(97)90215-7
12. Wang J, Zhang H, Fu S, Wang H, Ni D, Nasci R, et al. Isolation of Kyasanur forest disease virus from febrile patient, Yunnan, China. Emerg Infect Dis. 2009;15:326–8. DOI: 10.3201/eid1502.080979
13. Qattan I, Akbar N, Afif H, Abu Azmah S, Al-Khateeb T, Zaki A, et al. A novel flavivirus: Makkah Region, 1994–1996. Saudi Epidemiology Bulletin. 1996;3:1–3.
14. Charrel RN, Zaki AM, Attoui H, Fakeeh M, Billoir F, Yousef AI, et al. Complete coding sequence of the Alkhurma virus, a tick-borne flavivirus causing severe hemorrhagic fever in humans in Saudi Arabia. Biochem Biophys Res Commun. 2001;287:455–61. DOI: 10.1006/bbrc.2001.5610
15. Zhang TS, Wang YM, Zhang YH, Duan S. A survey of antibodies to arboviruses in residents of southwestern Yunnan Province [in Chinese]. Chinese Journal of Endemiology. 1989;10:74–7.
16. Hou ZL, Huang WL, Zi DY, Zhang HL, Shi HF. Study of the serologic epidemiology of tick-borne viruses in Yunnan [in Chinese]. Chinese Journal of Vector Biology and Control. 1992;3:173–6.
17. Zanotto PM, Gao GF, Gritsun T, Marin MS, Jiang WR, Venugopal K, et al. An arbovirus cline across the northern hemisphere. Virology. 1995;210:152–9. DOI: 10.1006/viro.1995.1326
18. Gould EA, de Lamballerie X, Zanotto PM, Holmes EC. Evolution, epidemiology, and dispersal of flaviviruses revealed by molecular phylogenies. Adv Virus Res. 2001;57:71–103. DOI: 10.1016/S0065-3527(01)57001-3
19. Gould EA, Solomon T. Pathogenic flaviviruses. Lancet. 2008;371:500–9. DOI: 10.1016/S0140-6736(08)60238-X
20. Zanotto PM, Gould EA, Gao GF, Harvey PH, Holmes EC. Population dynamics of flaviviruses revealed by molecular phylogenies. Proc Natl Acad Sci U S A. 1996;93:548–53. DOI: 10.1073/pnas.93.2.548
21. Katoh K, Kuma K, Toh H, Miyata T. MAFFT Version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 2005;33:511–8. DOI: 10.1093/nar/gki198
22. Galtier N, Gouy M, Gautier C. SeaView and Phylo_win, two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci. 1996;12:543–8.
23. Farris JS, Kallersjo M, Kluge AG, Bult C. Testing significance of incongruence. Cladistics. 1994;10:315–9. DOI: 10.1111/j.1096-0031.1994.tb00181.x
24. Swofford DL. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b 10. Sunderland (MA): Sinauer Associates; 2003.
25. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics. 1998;14:817–8. DOI: 10.1093/bioinformatics/14.9.817
26. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007;7:214. DOI: 10.1186/1471-2148-7-214
27. Charrel RN, Zaki AM, Fakeeh M, Yousef AI, de Chesse R, Attoui H, et al. Low diversity of Alkhurma hemorrhagic fever virus, Saudi Arabia, 1994–1999. Emerg Infect Dis. 2005;11:683–8.
28. Grard G, Moureau G, Charrel RN, Lemasson J-J, Gonzalez J-P, Gallian P, et al. Genetic characterization of tick-borne flaviviruses: new insights into evolution, pathogenetic determinants and taxonomy. Virology. 2007;361:80–92. DOI: 10.1016/j.virol.2006.09.015
29. Charrel RN, Fagbo S, Moureau G, Alqahtani MH, Temmam S, de Lamballerie X. Alkhurma hemorrhagic fever virus in *Ornithodoros savignyi* ticks. Emerg Infect Dis. 2007;13:153–5.
30. Baillie GJ, Kolokotronis SO, Waltari E, Maffei JG, Kramer LD, Perkins SL. Phylogenetic and evolutionary analyses of St. Louis encephalitis virus genomes. Mol Phylogenet Evol. 2008;47:717–28. DOI: 10.1016/j.ympev.2008.02.015
31. Bryant JE, Holmes EC, Barrett AD. Out of Africa: a molecular perspective on the introduction of yellow fever virus into the Americas. PLoS Pathog. 2007;3:e75. DOI: 10.1371/journal.ppat.0030075
32. Boshell J. Kyasanur Forest disease: ecologic considerations. Am J Trop Med Hyg. 1969;18:67–80.
33. Boshell JM, Rajagopalan PK. Observations on the experimental exposure to the monkeys, rodents and shrews to infestation of ticks in forest of Kyasanur forest disease area. Indian Journal of Medical Research. 1968;56:586–8.
34. Ali S, Ripley SD. Handbook of the birds of India and Pakistan. Vol 1–10. New York: Oxford University Press; 2002.

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Nurses' Contacts and Potential for Infectious Disease Transmission

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Nurses' contacts with potentially infectious persons probably place them at higher risk than the general population for infectious diseases. During an influenza pandemic, illness among nurses might result in staff shortage. We aimed to show the value of individual data from the healthcare sector for mathematical modeling of infectious disease transmission. Using a paper diary approach, we compared nurses' daily contacts (2-way conversation with >2 words or skin-to-skin contact) with those of matched controls from a representative population survey. Nurses (n = 129) reported a median of 40 contacts (85% work related), and controls (n = 129) reported 12 contacts (33% work related). For nurses, 51% of work-related contacts were with patients (74% involving skin-to-skin contact, and 63% lasted ≤15 minutes); 40% were with staff members (29% and 36%, respectively). Our data, used with simulation models, can help predict staff availability and provide information for pandemic preparedness planning.

During past influenza epidemics, hospital staff have been confronted with a surge of inpatients (*1–5*), and modeling studies predict collapse of the healthcare system if resources are not allocated carefully (*6*,*7*). To ensure the availability of healthcare during a pandemic, maintaining qualified staff capacity is crucial. Some pandemic preparedness plans therefore prioritize healthcare workers (HCWs) for preventive interventions, such as prophylaxis with antiviral drugs or vaccination (*8–10*). In addition to their indispensability, HCWs most likely are at higher risk than the general public for influenza because of their close interaction with infected patients and, presumably, more overall contacts.

Some simulation models use data on social contacts to account for disease spread (*11–15*). Models also have been used to assess the effectiveness of vaccinating HCWs against influenza in nursing homes (*16*). Several recently published studies reported contact rates for different population subgroups (*17–22*) and even representative population samples from different countries (*23–25*), but HCWs' contacts were not explicitly assessed. This lack of information curtails planning for pandemic preparedness. The current approach to identifying critical threshold parameters for a pandemic is to model disease spread in the population. However, the focus on the general population and an uncritical generalization of model parameters could potentially bias the assessment of disease spread and of available staff capacity within the healthcare sector. We aimed, therefore, to compare social contact data from nurses with data from matched controls in the general population.

Methods

We conducted a prospective contact survey of nurses in charge of inpatient care for 5 hospitals in the German federal state of Bavaria by using a paper diary approach. We compared the data with data from a matched sample of the German general population obtained in a previously conducted survey of contacts (*25*). In both surveys, a contact was defined as a 2-way conversation of >2 words (not by telephone) or skin-to-skin contact (*17*,*18*) as a surrogate for exposure to disease. Actual rates of disease transmission were not measured.

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DOI: 10.3201/eid1509.081475

Study Population

Nurse Sample

Hospitals in Germany are classified by level of care as basic, intermediate, and maximum. We selected 5 hospitals and 5 substitute hospitals representing the distribution of available hospital beds in different Bavarian regions and across the levels of care (*26*). If the head of a hospital refused consent for participation, we approached the head of a substitute hospital.

We included only nurses from the departments of internal medicine or surgery because these departments provide the majority of hospital beds in Germany. We assumed that during an influenza pandemic, most hospitalized persons would be admitted to these departments.

To equally represent work shifts (morning, afternoon, and night) and days of the week, we assigned combinations of work shifts and days for data collection to each hospital and department. Before the survey began, we visited the study hospitals and briefed the nurses on the study aims and methods. We asked the heads of the nursing departments to randomly select 1 of the nurses on duty during each assigned work shift on the assigned day. If the selected nurse refused to participate, another nurse from the same shift was randomly chosen. All nurses provided written informed consent before participating in the study.

We assumed a dropout rate of 20% of initial study participants and accounted for that proportion of declining participants. The calculated sample size for a normally distributed variable with a type I error of 5%, a type II error of 20%, and a difference of 5 in the mean number of contacts between nurses and an equally sized control group (SD = 15 in both groups) was adapted for nonparametric testing by a 15% increase (*27*). Hence, the estimated sample size for our study was 160 participants.

Matched Controls

We matched 1 control to each nurse by age (±3 years), sex, and day of data collection (Monday–Friday vs. the weekend [i.e., Saturday–Sunday]). Controls, who were not necessarily from Bavaria, originated from a contact survey of the German population conducted as part of the Improving Public Health Policy in Europe through the Modeling and Economic Evaluation of Interventions for the Control of Infectious Diseases (POLYMOD) project, which is described elsewhere (*25*). In brief, survey participants were recruited by an independent market research company. A representative household sample was selected by using a random-walk technique. In each household, the person with the birthday nearest the date was interviewed. After a face-to-face interview (multitheme survey), respondents filled out a contact diary during the following day. The diaries were collected in person. No incentives were given. We restricted the sample used for comparison with nurses to the summer round of the survey (May–July 2006) to correspond with the period of the survey of nurses. This subsample comprised 340 participants.

Data Collection

We used a modified version of both the questionnaire and the contact diary designed for the POLYMOD contact survey (*25*). We collected sociodemographic information about each study participant. Participants were asked to complete the diary during a 24-hour period starting at 5 AM on the assigned day. The diary was organized as a table in which participants recorded the following features of their contacts during work and leisure time: age (or age range) and sex of the contact person; location where the contact occurred (multiple locations possible); indication of whether physical contact was involved; and length of time the contact lasted. For each contact person, 1 row of the table had to be completed. If a participant had repeated contact with 1 person during the 24 hours of data collection, the characteristics of the contacts with this person were summarized. Additionally, we asked nurses to specify the contact person at work (patient, hospital staff, or other [e.g., visitor]) and, for contacts during travel, means of transportation (public or private).

If controls estimated their total number of contact persons at work to be >10, they were asked to report them aggregated, without specifying contact duration and other characteristics. If controls completed the diary on Monday–Friday, the aggregated contacts were added to those reported in the diary. Therefore, duration and other characteristics of controls' work contacts were not always available for analysis.

Data Analysis

We compared median number of contacts by using the Wilcoxon rank sum test or the Kruskal-Wallis 1-way analysis of variance. To compare numbers of contacts for nurses and controls, we used the Wilcoxon signed rank test for matched pairs. These analyses were completed with Stata 10.0 (StataCorp, College Station, TX, USA). In addition, to compare age mixing matrices, we grouped nurses, controls, and the persons they contacted by age and calculated mean number of contacts for each age group.

Results

Nurses

We selected 2 hospitals providing basic care, 1 providing intermediate care, and 2 providing maximum care. We replaced 1 of the selected hospitals providing maximum care because the head of the hospital did not consent to study participation. A total of 131 (82% response) nurses completed the diary during April–July 2007.

Nurses reported a median of 40 contacts (range 12–80) during 24 hours (Table 1). Total numbers of contacts did not differ by nurses' sex or by hospital department (surgery or internal medicine) or level of care. However, nurses had more contacts during Monday–Friday than during the weekend. A median of 34 contacts (range 3–66) were work related.

Nurses reported a total of 5,161 contacts. Most took place at work, more than half lasted ≤15 minutes, more than half involved skin-to-skin contact, and more than one third were with persons ≥60 years of age (Table 2). Ages of contact persons differed for work compared with other places. At work, most contacts were with persons ≥60 years of age; a small percentage was with persons <20 years. At other places, most contacts were with persons 20–39 years of age, and the proportion with persons <20 years was higher than the proportion at work.

Most work-related contacts were with patients (51%) or other staff members (40%); 9% were with other persons. Of those with patients, 74% involved skin-to-skin contact, and 63% lasted ≤15 minutes. Of contacts with staff members, 67% were nonphysical, and 62% lasted >15 minutes. Most contacts with other persons were nonphysical (79%) and lasted ≤15 minutes (87%) (Table 3).

Matched Comparison

We matched controls to 129 (98%) nurses; 2 nurses could not be matched because day of data collection was unknown. Twelve percent of controls were housewives or were unemployed or retired.

Matched nurses reported more total contacts than did controls (5,071 vs. 2,741; median 40 vs. 12; p<0.0001) and more contacts at work (4,288 vs. 1,996; median 34 vs. 4; p<0.0001) (Figure). In other locations, numbers of contacts were similar (783 vs. 745; both medians 5, p = 0.73). In both samples, more contacts occurred during Monday–Friday than during the weekend. Regardless of day of data collection (Monday–Friday or weekend), nurses had more contacts than did controls (Monday–Friday: total contacts median 41.5 vs. 21, p<0.0001; work median 35.5 vs. 16, p<0.0001; other, both medians 5, p = 0.79; on weekends: total contacts median 32 vs. 6, p<0.0001; work median 27 vs. 0, p<0.0001; other median 5 vs. 4, p = 0.85).

We calculated mean number of individually reported contacts by nurses' ages or controls' ages and by contact persons' ages (excluding those at work reported by controls only in aggregated manner) (Table 4). Whereas controls tended to interact with persons from their own age group, nurses interacted with persons from a wider variety of age groups, primarily because of the inclusion of older age groups among patients.

Discussion

Our findings correspond with the nature of contact between HCWs and patients. Nurses have a high frequency of close contact with patients, but the time they can dedicate to each patient is limited. This pattern differed fundamentally from that of their work contacts with staff members or other persons, the characteristics of which agreed with those described by Mossong et al. (*25*), who found that contact intensity (i.e., whether physical contact was involved) correlated positively with contact duration in a general population sample.

Contact data are important for modeling the spread of infectious diseases. Our results show that the use of general population data might lead to inaccurate modeling results for the healthcare sector because HCWs' patterns of con-

Table 1. Characteristics of 131 nurses surveyed to determine extent of work and other contacts, Bavaria, Germany, April–July 2007*

Characteristic	No. (%) nurses	Median no. reported contacts	p value
Sex			
Female	115 (88)	40	0.35
Male	16 (12)	41	
Leisure activities ≥1×/wk in group of ≥5 persons	68 (52)		
Use of public transportation			
Any	86 (66)		
Daily	7 (8)		
Day of diary completion			
Weekday (Monday–Friday)	96 (73)	41.5	<0.05
Weekend (Saturday/Sunday)	33 (25)	32	
Unknown	2 (2)		
Hospital department			
Internal medicine	60 (46)	40	0.46
Surgery	60 (46)	41.5	
Both	11 (8)		
Hospital level of care			
I (basic)	60 (46)	42	0.35
II (intermediate)	20 (15)	31.5	
III (maximum)	51 (39)	39	

*Mean age of nurses was 35 y (range 18–59 y). Mean number of household members was 2 (range 1–7).

Table 2. Characteristics of 5,161 contacts reported by surveyed nurses, Bavaria, Germany, April–July 2007

Characteristic	Reported contacts, no. (%)
Place of contact	
Work	4,207 (82)
Home	226 (4)
Transportation	32 (<1)
Other places	349 (7)
Multiple locations	232 (4)
Not specified	115 (2)
Contact duration	
≤15 minutes	2,650 (51)
>15–60 minutes	1,244 (24)
>1 hour	1,202 (23)
Not specified	65 (1)
Contact intensity	
Skin-to-skin	2,646 (51)
Not skin-to-skin	2,331 (45)
Not specified	184 (4)
Age of contact person, y	
<20	284 (6)
20–39	1,535 (30)
40–59	1,558 (30)
≥60	1,776 (34)
Not specified	8 (<1)
Age of person contacted at work, y*	
<20	160 (4)
20–39	1,171 (28)
40–59	1,264 (30)
≥60	1,611 (38)
Not specified	1 (<1)
Age of person contacted outside work, y†	
<20	94 (15)
20–39	226 (37)
40–59	204 (34)
≥60	83 (14)

*n = 4,207.
†n = 607.

tact with patients differ fundamentally from the day-to-day contacts of the general population. However, although contact data form the basis of simulation models, other parameters may modify the influence of contact patterns. Our data might change current modeling predictions with respect to 1) spread of disease within the nurse population that results in staff shortage and 2) spread of disease from nurses to the general population.

Our findings suggest that the risk for infectious diseases by airborne transmission might be greater for nurses than for the general population because of nurses' more frequent and more intensive professional contacts with potentially infected patients. In addition, nurses' high proportion of work-related skin-to-skin contact highlights the potential for fecal–oral transmission of disease (e.g., norovirus infection).

Whether increased risk for infection in nurses during an influenza pandemic would lead to an earlier peak in the number of infections in HCWs than in the general population needs to be investigated in modeling studies. During a pandemic, maintaining sufficient staff during peak hospitalizations of the general population will be crucial. Our data may help guide public health interventions to prevent the infections in hospital staff and in the general population from peaking simultaneously.

Nurses most likely would take preventive measures, such as wearing personal protective equipment, when handling patients with clinically manifested influenza. However, their risk for infection from patients hospitalized for other reasons and already infected with influenza, but not yet clinically ill or with influenza diagnosis, might be more important for initial spread of disease in the healthcare sector. We collected our data during the interpandemic period, so we cannot predict how HCWs' contact behavior might change during a pandemic. Models using our data will need to account for this uncertainty.

Nurses can potentially spread infection from infected patients to the general population. In Germany, nurses represent 0.5% of the population (*28*). After infection is introduced into the nurse population, further contact with patients, other staff members, and relatives might result in faster spread to the general population during the early phase of a pandemic. The combination of contact with persons from older age groups at work and from younger age groups at home might facilitate spread among different age groups that would not otherwise occur.

These points support the need to add a separate healthcare component to models, i.e., modeling both HCWs and general population by using self-reported contact data instead of assuming similar contact rates for nurses and the general population. Additional data are required for modeling studies to determine the extent to which nurses and patients would respectively benefit from vaccination of HCWs against influenza.

Our contact definition captures only the amount of social contact with other persons. Because we did not measure rates of transmission or disease, use of our data for modeling relies on the assumption that the number of contacts correlates with the amount of exposure to disease.

Regarding the ongoing discussion about the major route of transmission of influenza (*29*,*30*), we might overestimate exposure to disease by counting conversational contacts if influenza is transmitted predominantly by droplet spread. On the other hand, accounting only for skin-to-skin contact might considerably underestimate exposure because, among other reasons, transmission by contaminated surfaces or fomites would not be included. To provide data for modeling the spread of influenza and other infectious diseases with various routes of transmission, we dichotomized the data.

Furthermore, for nurses, the distribution of conversational and skin-to-skin contacts differs among contacts

Table 3. Work-related contacts of nurses, Bavaria, Germany, April–July 2007*

Contact duration, min	No. nonphysical contacts with				No. of physical contacts with			
	Patients	Staff	Other	All	Patients	Staff	Other	All
≤15	441	507	279	1,227	927	114	37	1,078
15–60	69	332	12	413	483	135	10	628
>60	7	319	5	331	226	247	17	490
Total	517	1,158	296	1,971	1,636	496	64	2,196

*Only contacts with information on all 3 variables (n = 4,167).

with patients and with others. Consequently, different contact matrices should be used for modeling when different transmission probabilities are assumed for conversational and skin-to-skin contacts.

Our study has some limitations. First, our nurse sample might not be entirely random because potential participants could refuse participation. If nurses willing to participate in the study had more contacts than nurses not willing to participate, our results could overestimate nurses' contact numbers. Second, nurses' motivation to participate might have been higher than that of controls, resulting in more reported contacts; a reason for nurses' increased motivation might be our visits to study hospitals to inform nurses about the aims of the study and the potential benefits of participating for their occupation. The method used to recruit controls from the POLYMOD study required conduct of the survey in study participants' homes. Even though home visits were timed to minimize this possible selection bias, the sampling still might have resulted in a higher proportion of housewives and unemployed and retired persons, all of whom may have fewer contacts than the general population. However, because patients' needs largely determine the nurses' number of work contacts and because the numbers of non–work-related contacts were comparable for nurses and controls, we do not expect that a large selection bias accounts for the difference.

A third potential limitation is the difference in work patterns of nurses and controls. Nurses worked on days they were surveyed, even on weekends, and controls might not have had work contacts during some days of diary completion. This difference is regarded, not as bias, but as potentially meaningful information that needs to be considered when modeling nurses' contacts. Still, the separate analysis by day of the week persistently differed between the groups. The difference in work contacts reported by nurses and controls also might be affected by controls' reporting of aggregated work contacts instead of single diary entries if they usually had >10 contacts at work. Not considering these aggregated work contacts for weekend days might have underestimated controls' contact numbers. However, again, the separate comparison by day points to more contacts of nurses than controls. Evidence conflicts about whether disaggregated contact reporting is more complete than aggregated reporting. One study showed higher median contact numbers comparing prospective to retrospective reporting among university students (*22*). By contrast, another group that compared a diary approach with a 1 time-point estimation of contacts decided in favor of aggregated contacts because most diaries were completed retrospectively (*31*). However, most respondents in the POLYMOD sample from Germanyof the POLYMOD study (*25*) stated that they completed the diary during the day and not retrospectively. The large difference in work contacts between nurses and controls is difficult to explain solely by different methods of contact reporting.

Finally, we perhaps missed types of contacts that do not include conversation or physical contact. Missed contacts might include crowds (e.g., during public transportation), which have been discussed in the context of airborne spread of infections (*29*,*30*). However, the average risk associated with a conversation or with physical contact can be reasonably assumed to be substantially higher than just presence in the same room.

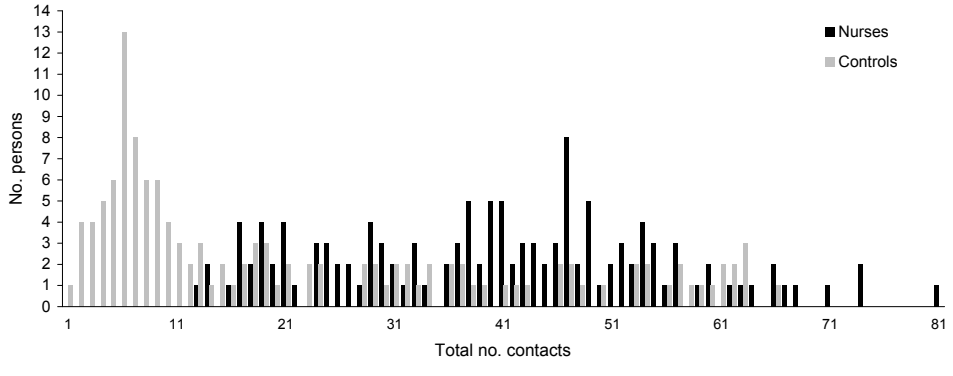

Figure. Total numbers of contacts for surveyed nurses and their matched controls from the general population, Bavaria, Germany, April–July 2007.

Table 4. Mean number of individually reported contacts between nurses or controls and other persons, by age group, Bavaria, Germany, April–July 2007*

Age group, y	No. nurses/ controls	Mean no. contacts by age group of contact person									
		0–4 y	5–14 y	15–24 y	25–34 y	35–44 y	45–54 y	55–64 y	65–74 y	75–84 y	85–94 y
Nurses											
15–24	32	0.00	0.13	2.91	4.09	2.94	2.53	2.22	3.00	2.88	0.81
25–34	29	0.28	0.55	6.21	9.31	9.69	7.24	6.79	5.97	5.17	1.45
35–44	31	0.29	1.03	4.13	4.74	6.16	6.13	3.68	5.81	4.94	1.23
45–54	30	0.30	0.63	3.47	5.03	6.40	5.93	3.90	3.87	2.83	1.13
55–64	7	0.29	0.29	2.71	4.57	5.43	3.29	3.43	4.43	3.86	0.43
Controls											
15–24	32	0.00	0.29	6.10	2.38	1.43	1.71	0.76	0.14	0.14	0.00
25–34	29	0.29	0.45	0.60	1.48	1.17	0.74	0.64	0.14	0.07	0.02
35–44	31	0.13	0.26	1.29	1.00	2.29	1.48	0.52	0.42	0.26	0.00
45–54	30	0.07	0.18	0.86	0.96	1.61	1.93	0.71	0.36	0.25	0.00
55–64	7	0.00	0.57	0.57	1.43	0.71	1.86	1.71	0.43	0.14	0.00

*Total number of contacts was 5,071 for nurses and 993 for controls. Controls were not all from Bavaria.

Hospital structure and tasks of nurses in charge of inpatient care are identical in the different German federal states. Therefore, we expect our results from hospitals in the German federal state of Bavaria to be representative of all of Germany. However, because structures and standards of care might differ in other countries, our data require country-specific validation. Furthermore, patterns of contact for hospital workers other than nurses in charge of inpatient care (e.g., physicians, technical assistants, cleaning personnel, or nurses in emergency departments) might differ substantially and influence the patterns of infectious disease spread in the hospital. However, because most routine daily patient contacts are with inpatient nurses, we are confident our data reflect the pattern of a large proportion of contacts in German hospitals.

Our survey did not account for length of hospital stay. Repeated contacts with long-term patients might not bear the same risk for infection as contacts with newly admitted patients because long-term patients are less likely to import an infection to the hospital. Future modeling studies might investigate the impact of length of hospital stay on disease spread.

In our study, nurses' patterns of contact differed from those of the general population. Our findings support the need for explicit modeling of the healthcare sector to assess the spread of epidemics. To this aim, our study provides quantitative estimates of contact patterns. On the basis of results of modeling approaches for the healthcare sector, public health policies should be reassessed and revised as necessary.

Acknowledgments

We thank the nurses from the 5 study hospitals for participating in this survey. We also thank Martin Eichner, Wolfgang Hautmann, Maria-Sabine Ludwig, and Markus Schwehm for stimulating discussions; Christiane Hoeller and Andreas Sing for their advice in the conceptual phase of the study; Katharina Alpers, Viviane Bremer, Udo Buchholz, and Martin Eichner for helpful comments on the study protocol; the participants of the 2008 POLYMOD meeting and of the 2008 European Program for Intervention Epidemiology Training project review module for fruitful discussions of study results; and Udo Buchholz and Doris Radun for helpful comments on the manuscript.

The work of H.B. was financed by an influenza research grant provided by the Federal Ministry of Education and Research and the Federal Ministry of Health. Contact data on the matched controls were collected in the framework of the POLYMOD project. POLYMOD was financially supported by grant SP22-CT-2004-502084 of the European Commission, DG Research.

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References

1. Glaser CA, Gilliam S, Thompson WW, Dassey DE, Waterman SH, Saruwatari M, et al. Medical care capacity for influenza outbreaks, Los Angeles. Emerg Infect Dis. 2002;8:569–74.
2. Andreasen V, Viboud C, Simonsen L. Epidemiologic characterization of the 1918 influenza pandemic summer wave in Copenhagen: implications for pandemic control strategies. J Infect Dis. 2008;197:270–8. DOI: 10.1086/524065
3. Gani R, Hughes H, Fleming D, Griffin T, Medlock J, Leach S. Potential impact of antiviral drug use during influenza pandemic. Emerg Infect Dis. 2005;11:1355–62.
4. Kawana A, Naka G, Fujikura Y, Kato Y, Mizuno Y, Kondo T, et al. Spanish influenza in Japanese armed forces, 1918–1920. Emerg Infect Dis. 2007;13:590–3.
5. Nguyen-Van-Tam JS, Hampson AW. The epidemiology and clinical impact of pandemic influenza. Vaccine. 2003;21:1762–8. DOI: 10.1016/S0264-410X(03)00069-0
6. Nap RE, Andriessen MP, Meessen NE, van der Werf TS. Pandemic influenza and hospital resources. Emerg Infect Dis. 2007;13:1714–9.
7. Nap RE, Andriessen MP, Meessen NE, Miranda DR, van der Werf TS. Pandemic influenza and excess intensive-care workload. Emerg Infect Dis. 2008;14:1518–25. DOI: 10.3201/eid1410.080440

8. Straetemans M, Buchholz U, Reiter S, Haas W, Krause G. Prioritization strategies for pandemic influenza vaccine in 27 countries of the European Union and the Global Health Security Action Group: a review. BMC Public Health. 2007;7:236. DOI: 10.1186/1471-2458-7-236
9. Mounier-Jack S, Coker RJ. How prepared is Europe for pandemic influenza? Analysis of national plans. Lancet. 2006;367:1405–11. DOI: 10.1016/S0140-6736(06)68511-5
10. WHO global influenza preparedness plan. Pandemic influenza preparedness and response: a WHO guidance document [cited 2009 Jun 29]. Available from http://www.who.int/csr/disease/influenza/PIPGuidance09.pdf
11. Longini IM Jr, Nizam A, Xu S, Ungchusak K, Hanshaoworakul W, Cummings DA, et al. Containing pandemic influenza at the source. Science. 2005;309:1083–7. DOI: 10.1126/science.1115717
12. 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
13. 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
14. Bansal S, Pourbohloul B, Meyers LA. A comparative analysis of influenza vaccination programs. PLoS Med. 2006;3:e387. DOI: 10.1371/journal.pmed.0030387
15. Eichner M, Schwehm M, Duerr HP, Brockmann SO. The influenza pandemic preparedness planning tool InfluSim. BMC Infect Dis. 2007;7:17. DOI: 10.1186/1471-2334-7-17
16. van den Dool C, Bonten MJ, Hak E, Heijne JC, Wallinga J. The effects of influenza vaccination of health care workers in nursing homes: insights from a mathematical model. PLoS Med. 2008;5:e200. DOI: 10.1371/journal.pmed.0050200
17. Mikolajczyk RT, Akmatov MK, Rastin S, Kretzschmar M. Social contacts of school children and the transmission of respiratory-spread pathogens. Epidemiol Infect. 2008;136:813–22. DOI: 10.1017/S0950268807009181
18. Edmunds WJ, O'Callaghan CJ, Nokes DJ. Who mixes with whom? A method to determine the contact patterns of adults that may lead to the spread of airborne infections. Proc Biol Sci. 1997;264:949–57. DOI: 10.1098/rspb.1997.0131
19. Edmunds WJ, Kafatos G, Wallinga J, Mossong JR. Mixing patterns and the spread of close-contact infectious diseases. Emerg Themes Epidemiol. 2006;3:10. DOI: 10.1186/1742-7622-3-10
20. Beutels P, Shkedy Z, Aerts M, Van DP. Social mixing patterns for transmission models of close contact infections: exploring self-evaluation and diary-based data collection through a web-based interface. Epidemiol Infect. 2006;134:1158–66. DOI: 10.1017/S0950268806006418
21. Wallinga J, Edmunds WJ, Kretzschmar M. Perspective: human contact patterns and the spread of airborne infectious diseases. Trends Microbiol. 1999;7:372–7. DOI: 10.1016/S0966-842X(99)01546-2
22. Mikolajczyk RT, Kretzschmar M. Social contact data in the context of disease transmission: prospective and retrospective study designs. Soc Networks. 2008;30:127–35. DOI: 10.1016/j.socnet.2007.09.002
23. Fu Y. Measuring personal networks with daily contacts: a single-item survey question and the contact diary. Soc Networks. 2005;27:169–86. DOI: 10.1016/j.socnet.2005.01.008
24. Wallinga J, Teunis P, Kretzschmar M. Using data on social contacts to estimate age-specific transmission parameters for respiratory-spread infectious agents. Am J Epidemiol. 2006;164:936–44. DOI: 10.1093/aje/kwj317
25. Mossong J, Hens N, Jit M, Beutels P, Auranen K, Mikolajczyk R, et al. Social contacts and mixing patterns relevant to the spread of infectious diseases. PLoS Med. 2008;5:e74. DOI: 10.1371/journal.pmed.0050074
26. Bayerisches Staatsministerium für Arbeit und Sozialordnung. Familie und Frauen—Krankenhausplan des Freistaates Bayern—32. Fortschreibung. Munich (Germany): Bavarian State Ministry of Labor and Social Welfare, Family Affairs and Women; 2007.
27. Lehmann EL. Nonparametrics: statistical methods based on ranks, revised. San Francisco: Holden-Day; 1975.
28. Federal Statistical Office Germany [cited 2009 Jul 6]. Available from http://www-genesis.destatis.de/genesis/online/online
29. Brankston G, Gitterman L, Hirji Z, Lemieux C, Gardam M. Transmission of influenza A in human beings. Lancet Infect Dis. 2007;7:257–65. DOI: 10.1016/S1473-3099(07)70029-4
30. Tellier R. Review of aerosol transmission of influenza A virus. Emerg Infect Dis. 2006;12:1657–62.
31. Glass LM, Glass RJ. Social contact networks for the spread of pandemic influenza in children and teenagers. BMC Public Health. 2008;8:61. DOI: 10.1186/1471-2458-8-61

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Genetics and Pathogenesis of Feline Infectious Peritonitis Virus

Meredith A. Brown, Jennifer L. Troyer, Jill Pecon-Slattery, Melody E. Roelke, and Stephen J. O'Brien

Feline coronavirus (FCoV) is endemic in feral cat populations and cat colonies, frequently preceding outbreaks of fatal feline infectious peritonitis (FIP). FCoV exhibits 2 biotypes: the pathogenic disease and a benign infection with feline enteric coronavirus (FECV). Uncertainty remains regarding whether genetically distinctive avirulent and virulent forms coexist or whether an avirulent form mutates in vivo, causing FIP. To resolve these alternative hypotheses, we isolated viral sequences from FCoV-infected clinically healthy and sick cats (8 FIP cases and 48 FECV-asymptomatic animals); 735 sequences from 4 gene segments were generated and subjected to phylogenetic analyses. Viral sequences from healthy cats were distinct from sick cats on the basis of genetic distances observed in the membrane and nonstructural protein *7b* genes. These data demonstrate distinctive circulating virulent and avirulent strains in natural populations. In addition, 5 membrane protein amino acid residues with functional potential differentiated healthy cats from cats with FIP. These findings may have potential as diagnostic markers for virulent FIP-associated FCoV.

Feline infectious peritonitis (FIP) is an uncommon, fatal, progressive, and immune-augmented disease of cats caused by feline coronavirus (FCoV) infection. Although FCoV is common in most domestic, feral, and nondomestic cat populations worldwide (seroprevalence 20%–100%), FIP will develop in <10% of FCoV seropositive cats (*1–4*). FIP tends to occur most frequently in cats <2 years of age or, less commonly, in geriatric cats (*4,5*). The clinical manifestations of FCoV infection can be either a pathogenic disease, FIP (cases infected with feline infectious peritonitis virus [FIPV]) and, more commonly, a benign, or mild enteric infection (feline enteric coronavirus [FECV] asymptomatic) (*6,7*). Specific genetic determinants of these clinical outcomes have yet to be discovered. There is no effective treatment, vaccine, or diagnostic protocol that can discriminate the avirulent FECV from FIPV.

FIP pathology is characterized typically by severe systemic inflammatory damage of serosal membranes and widespread pyogranulomatous lesions, which occurs in the lungs, liver, lymph tissue, and brain (*8*). Evidence suggests that the host immune system is crucial in this pathogenesis; profound T-cell depletion from the periphery and lymphatic tissues and changes in cytokine expression are observed in end-stage FIP (*9,10*). The clinical finding of hypergammaglobulinemia-associated FIP is indicative of virus-induced immune dysregulation (*11*).

Viral genetic determinants specifically associated with FIPV pathogenesis have yet to be discovered. An in vivo mutation transition hypothesis postulated that de novo virus mutation occurs in vivo, giving rise to virulence (*12,13*). The precise nature of the mutation responsible for pathogenesis has not been identified, although studies have suggested sequence differences in the spike protein (*14*), nonstructural protein (NSP) 7b, and NSP3c (*13*) as disease determinants. Together with in vitro studies describing the FIPV strains affinity for macrophages in contrast to FECV strains (*15*), the hypothesis was extended to propose that the enteric coronavirus (FECV) undergoes a mutational shift in the gastrointestinal system, thus allowing infection of macrophages, systemic dissemination, and fatal disease manifestation (*12,13*). However, attempts to use engineered chimeric viruses designed to identify the operative virulence determinants have been unsuccessful (*16*). Furthermore, circulating FCoVs found in different tissues of FCoV-infected asymptomatic cats were indistinguishable (*17,18*).

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DOI: 10.3201/eid1509.081573

The in vivo mutation hypothesis of FIPV pathogenesis is widely cited, although it has never been explicitly confirmed. Mutational transition of HIV-1 has been demonstrated in AIDS patients, in which mutation of envelope residues alters coreceptor use from CCR5 to CXCR4, a prelude to the collapse of the CD4-bearing lymphocyte population (*19*). Similarly, key amino acid changes in the porcine coronavirus spike gene lead to increased virulence in the coronavirus transmissible gastroenteritis virus, a fatal disease causing high rates of illness and death in young pigs (*20–22*).

An alternative circulating virulent-avirulent FCoV hypothesis of viral pathogenesis suggests that distinctive benign and pathogenic strains of FECV circulate in a population, and that the disease will develop only in those persons infected by the virulent strains. Dengue virus may offer an example because it has been shown that 4 distinctive viral strains circulate worldwide, and severe hemorrhagic fever develops in persons exposed sequentially to distinct strains (*23*). Zoonotic equine Venezuelan encephalitis virus also displays circulating virulent and avirulent strains, which through interaction with ecologic and epidemiologic factors, contribute to or constrain the disease incidence (*24*).

This study aimed to systematically test evolutionary predictions of the in vivo mutation hypothesis versus the circulating virulent/avirulent hypothesis in the pathogenicity of FIP in the cat. We developed a study of naturally occurring FECV and FIPV using molecular genetic tools by collecting samples from field cases of FIP (cases) and FECV-positive but asymptomatic cats (controls). Cases were infected with feline coronavirus (FCoV) and had the clinical disease of feline infectious peritonitis (FIP). Controls were also infected with FCoV, but were clinically asymptomatic (FECV-asymptomatic). The prediction was that phylogenetic analysis of viral gene sequences would demonstrate paraphyly for FIP case-cats and FECV-asymptomatic cats if the in vivo mutation hypothesis was supported, and monophyly of the 2 if the circulating virulent/avirulent hypothesis was supported (Figure 1). Additionally, we surveyed the viral genetic diversity and dynamics and determined genetic signatures associated with pathogenesis in FIP.

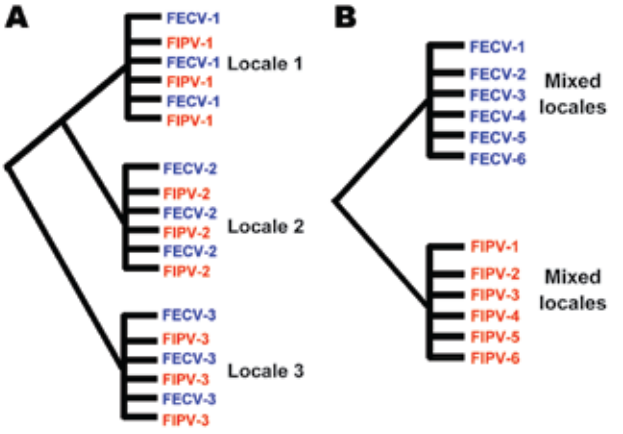


Figure 1. Alternative phylogenetic predictions of the in vivo mutation hypothesis versus the dual circulating virulent/avirulent hypothesis. A) The in vivo mutation transition hypothesis predicts paraphyly of feline infectious peritonitis (FIP) cases and feline enteric coronavirus (FECV) asymptomatic feline coronavirus (FCoV) isolates). B) The circulating virulent/avirulent strain hypothesis predicts reciprocal monophyly of FIV-cases versus FECV asymptomatic. Numbers represent individual cat (or locale), which is either FIPV case (red) or FECV asymptomatic (blue). Evidence presented in this article supports the circulating dual virulent and avirulent strains.

Materials and Methods

Sampling

A total of 56 live, euthanized, or recently deceased domestic cats were clinically examined and sampled in Maryland veterinary hospitals during 2004–2006 (online Appendix Table 1, available from www.cdc.gov/EID/content/15/9/1445-appT1.htm). Blood (3–6 mL) was collected routinely by venipuncture from manually restrained or anesthetized domestic cats. Feces were obtained from the rectum by cotton swab and frozen in 0.5 mL of phosphate-buffered saline. Cats from 1 (Weller Farm) of 6 farms were micro-chipped (AVID, Folsom, LA, USA) for identification for repeat sampling of individual cats. Samples were collected in full compliance with specific federal permits (Convention on International Trade in Endangered Species; Endangered and Threatened Species) issued to the National Cancer Institute by the US Fish and Wildlife Service of the Department of the Interior.

For euthanized and recently deceased cats, gross necropsy examination and sample collection were performed within 2 hours of death. Samples from liver, spleen, mesenteric lymph node, kidney, jejunum, and colon were taken, fixed in 10% buffered formalin, and routinely embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (HE). Tissues were also flash frozen in liquid nitrogen (–220°C) for RNA extraction and stored at either –220°C or –70C°.

Clinical Hematologic and Biochemical Analysis

For complete blood counts, fresh (<12 hours) whole-blood samples were assessed by Antech veterinary diagnostic laboratory by using an automated cell counter (Avid Cell-Dyn 3500; Abbott Laboratories, Abbott Park, IL, USA). Biochemical analysis (Hitachi 717 Clinical Chemistry Analyzer; Roche Diagnostics, Indianapolis, IN, USA) and ELISA for feline immunodeficiency virus (FIV; Petchek FIV ELISA, Idexx Laboratories, Westbrook, ME,

USA), and coronavirus (Virachek CV, Synbiotics Corp., San Diego, CA, USA) antibodies were also performed.

Pathologic and Immunohistochemical Analysis

HE-stained slides of spleen, liver, lymph node, intestine, and kidney sections were evaluated for evidence of granulomatous and pyogranulomatous lesions (National Cancer Institute Laboratory Animal Sciences Program, Frederick, MD, USA). Formalin-fixed sections (3 μm thick) were cut from paraffin blocks and placed on glass slides for immunohistochemical (IHC) testing, as previously described, with CoV p56, a cross-reacting antibody for the demonstration of feline coronavirus (FECV and FIPV biotypes) (*9,10*) (Washington Animal Disease Diagnostic Laboratory, Pullman, WA, USA) (Figure 2).

RNA Extraction and Reverse Transcription

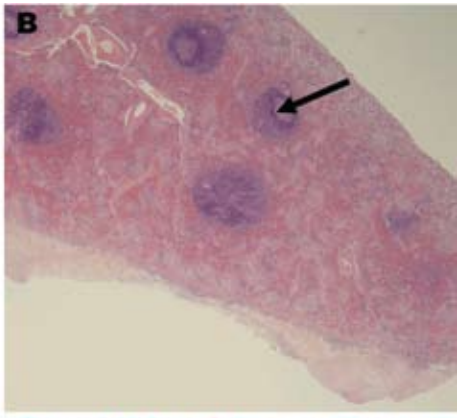
RNA from 160 μL ascites fluid or frozen feces suspended 10% in phosphate-buffered saline was extracted by using the QIAamp virus RNA mini kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. RNA from tissue was extracted from ≈60 mg of frozen liver, lung, spleen, colon, jejunum, or lymph node by using RNAeasy (QIAGEN) following the manufacturer's instructions. Extracted RNA was eluted in 35 μL of RNase-free water and stored at –70ºC. cDNA was reverse transcribed using 9 μL of eluted RNA (10 pg–5 μg) in an initial 12-μL reaction mixture containing 50 ng of random hexamers and 0.5 mmol/L of dNTPs. After incubation at 65ºC for 5 min to denature the RNA, 10 mmol/L of dithiothreitol, 5× Synthesis Buffer, 40 U of RNaseOUT, and 15 units of Thermoscript RT were added on ice (Invitrogen, Carlsbad, CA, USA). Reaction mixtures were incubated in thermocycler at 25ºC for 10 min, followed by 50ºC for 30 min. cDNA was stored at –20ºC.

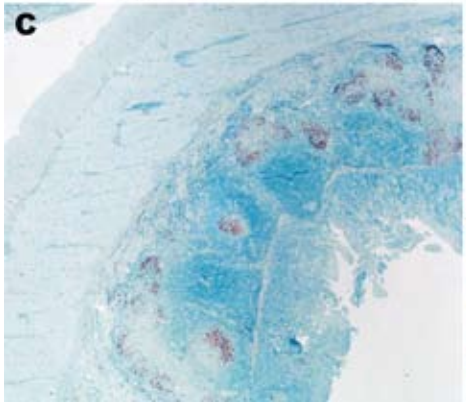
PCR

Primers amplifying 7b (736 bp), membrane protein (575 bp), polymerase (386 bp), and spike-NSP3 (1,017 bp) (Figure 3) were designed based on available feline coronavirus sequence (*1,12,13*). PCR was performed by using 2 μL of cDNA in a 50-μL reaction containing 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 0.25 mmol/L concentrations of dNTPs (dATP, dCTP, dGTP, and dTTP), 2 mmol/L concentrations of each primer, and 2.5 units of Platinum Taq DNA polymerase (Invitrogen). PCR was conducted on a geneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following touchdown conditions: 2 min at 94ºC then a touchdown, always starting with 20 sec at 94ºC, then 30 sec at 60ºC (3 cycles), 58ºC (5 cycles), 56ºC (5 cycles), 54ºC (5 cycles), 52ºC (22 cycles), and then 1 min at 72ºC for extension, and with a final extension at 72ºC for 7 min and a hold at 4ºC. PCR products were visualized by

A

Cat ID no.	Histopathology	IHC
Fca-4549	Pos	Liver, colon, jejunum
Fca-4554	Neg	Neg
Fca-4561	Neg	jejunum
Fca-4562	Neg	Neg
Fca-4563	Neg	Neg
Fca-4564	Neg	Neg
Fca-4586	Pos	Liver, spleen, lymph node
Fca-4590	Pos	Lung, jejunum, lymph node
Fca-4618	Pos	Liver, lymph node
Fca-4620	Neg	Neg
Fca-4623	Neg	Neg
Fca-4624	Neg	Neg
Fca-4625	Neg	Neg
Fca-4653	Pos	Liver, spleen, colon
Fca-4655	Neg	Neg
Fca-4656	Neg	Neg
Fca-4657	Neg	Neg
Fca-4658	Neg	Neg
Fca-4659	Neg	Neg
Fca-4660	Neg	Neg
Fca-4662	Pos	ND
Fca-4663	Pos	ND
Fca-4664	Pos	ND





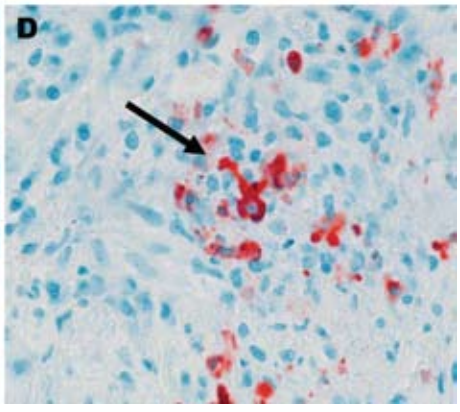
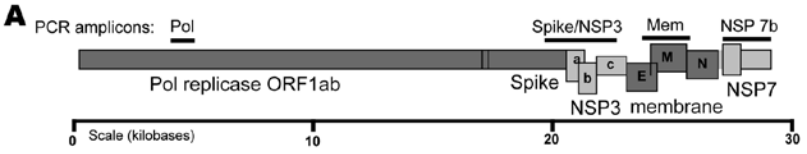


Figure 2. A) Histopathologic and immunohistochemical (IHC) results from 23 necropsied cats positive for antibodies against feline coronavirus. Liver, lung, spleen, colon, jejunum, stomach, heart, kidney, lymph node were evaluated by IHC. Feline infectious peritonitis (FIP) cases are highlighted in gray. Pos, positive; Neg, negative; ND, not done. B) Representative tissues from cat no. FCA-4653, spleen (histopathologic) showing granuloma (arrow); magnification ×20. C) Representative tissues from cat no. FCA-4590, small intestine (IHC); magnification ×20. D) Red staining indicates binding of coronavirus antibody (CoV p56, arrow); magnification ×100.



B

Gene	Primer sequences		Length, bp	No. cats positive for		No. clone sequences (no. unique clones)	
	Forward	Reverse		FECV	FIPV	FECV	FIPV
Pol replicase	CTCACAAGTTATACTCAAGGGC	TCAGTGGTTTACCAGCTTGCA	384	9	5	47 (22)	70 (37)
Spike, NSP 3a-c	AATGGCCTTGGTATGTGTGG	CCTGAGAAAAGGCTGCATTGT	1,017	9	7	20 (20)	28 (23)
Membrane	TCTTGCTAACTGGAACTTCAGCTGG	TGACGCGTTGTCCCTGTGTG	575	21	8	135 (98)	118 (65)
NSP 7b	GGGTTTTCCTGCTATACATTG	CACTAGATCCAGACGTTAGCTC	736	19	8	164 (131)	153 (105)
					Total	366 (271)	369 (230)

Figure 3. A) Feline coronavirus genome indicating PCR products obtained (bars). Structural proteins are shaded in dark gray; nonstructural proteins are shaded in light gray. B) Forward and reverse primers used to amplify virus segments are listed in 5′ → 3′ orientation. The number of source cats and cloned sequences generated from feline infectious peritonitis (FIP) cases and feline enteric coronavirus (FECV) asymptomatic cats are presented. Pol, polymerase; NSP, nonstructural protein; FIPV, feline infectious peritonitis virus.

electrophoresis on a 1% agarose gel and primers and unincorporated dNTPs were removed by using Microcon YM (Millipore, Billerica, MA, USA).

Cloning and Sequencing

Representative PCR products were cloned and sequenced (Figure 3, panel B). Cloning was performed by using a TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was isolated from 1–47 clones from each reaction product (Agencourt CosMCPrep; Agencourt Bioscience Corporation, Beverly, MA, USA). The presence of the correct sized insert was verified by restriction enzyme digestion (*Eco*RI), and sequences were obtained from clones with the correct insert by using standard ABI BigDye terminator reactions (Applied Biosystems). Anticontamination measures were taken at all steps of reverse transcription–PCR (RT-PCR) amplification and post-PCR processing.

Phylogenetic Analysis

Sequences from *pol 1a, spike-NSP3, membrane,* and *NSP7b* were analyzed separately. Nucleotide sequences were compiled and aligned for subsequent phylogenetic analysis by using ClustalX (*25*) and verified visually (*26*). Analyses involved producing a phylogenetic tree of viral gene sequences based upon the following approaches: minimum evolution, maximum parsimony, and maximum likelihood in PAUP (*27*). Modeltest was used to estimate the optimal model of sequence evolution, and these settings were incorporated into subsequent analyses (*28*). Minimum evolution trees were constructed from models of substitution specified by Modeltest, with starting trees obtained by neighbor joining followed by application of a tree-bisection-reconnection (TBR) branch-swapping algorithm during a heuristic search for the optimal tree. Maximum parsimony analysis employed a heuristic search of starting trees obtained by stepwise addition followed by TBR. Maximum-likelihood parameters specified by Modeltest selected the general time-reversible model of substitution, included empirical base frequencies, and estimated rate matrix and corrected for among-site rate variation (gamma distribution). A bootstrap analysis using 1,000 iterations was performed for maximum parsimony and minimum evolution and 100 iterations by using the nearest neighbor interchange branch-swapping algorithm for maximum likelihood. Amino acid residue alignments were generated using MacClade 3.05 (*26*) and ClustalX (www.softpedia.com/get/Science-CAD/Clustal-X.shtml).

Variable sites and parsimoniously informative sites were computed in MEGA version 3.0 (*29*). Pairwise comparisons of genetic distances were performed in PAUP and the mean and range of genetic distances were calculated in Excel (Microsoft, Redmond, WA, USA). The sequences of FCoV *pol 1a, membrane, NSP 7b,* and *spike-NSP3* were deposited in GenBank under accession nos. EU663755–EU664317.

Results

During 2004–2006, fifty-six domestic cats with suspected FIP or exposure to infected FIP cats from Maryland farms and veterinary hospitals were sampled (online Appendix Table 1). All samples producing RT-PCR products were from cats positive for antibodies against FCoV (titers >25). Thirty-six sampled cats were from the Weller farm where several individual cats were sampled once per year for the 3-year study period. Healthy and recently deceased or euthanized cats were included from the Ambrose farm (n = 7), Palmer Veterinary Hospital (n = 3), Frederick County Animal Shelter (n = 7), Seymour farm (n = 1), and the New Market Animal Hospital (n = 2). Fca-4590 from the Weller farm is an important FIP case because samples were obtained on May 20, 2004, when the cat was clinically healthy (predisease) and again on December 22, 2004, when FIP developed in the cat and it died (postdisease).

Necropsies were performed on 23 cats that died or were euthanized due to suspected FIP. Most of the necropsied cats were FCoV antibody positive (online Appendix Table 1). Eight cats were classified as FIP cases based on histopatholgic findings (Fca-4549, Fca-4566, Fca-4590, Fca-4618, Fca-4653, Fca-4662, Fca-4663, and Fca-4664)

(Figure 2; online Appendix Table 1). The presence of pyogranulomatous lesions at histology evaluation was sufficient for designation of an FIP case. Additionally, 5 of the 8 FIP cases were evaluated by IHC testing. Multiple tissues were positive by IHC in each of these cats. One cat (Fca-4561) was IHC positive only in the jejunum and negative by histopathologic analysis on all tissues, therefore it was classified as FECV asymptomatic. The FCoV-seropositive necropsied cats with no characteristic FIP histopathologic changes and IHC lesions were classified as FECV asymptomatic (online Appendix Table 1; Figure 2). Healthy cats were classified as FECV asymptomatic if they had normal results on physical examinations, were FCoV antibody positive (titer >25) but not lymphopenic (<1.5 cells/μL), or were monitored until 2007 and known to be free of FIP disease (online Appendix Table 1).

RT-PCR was attempted with 4 primer pairs designed from FCoV genes for all cats (Figure 3, panel B). Of the 82 samplings from 56 cats, 42 samplings amplified virus with at least 1 primer pair yielding a 51% rate of recovery of viral sequence (online Appendix Table 1). From 8 cats with clinical FIP and 23 FECV-asymptomatic cats, amplification from the 4 viral regions produced 735 cloned viral gene segments that resulted in 501 unique gene sequences (online Appendix Table 2, available from www.cdc.gov/EID/content/15/9/1445-appT2.htm; Figure 3, panel B).

Phylogenetic analysis of the cloned virus sequences from 3 Maryland locales sampled during 2004–2006 showed specific patterns of viral dynamics. First, gene sequences from healthy cats infected with FECV displayed a monophyletic cluster pattern that was generally distinctive from cats diagnosed with FIP in the *membrane*, *NSP 7b,* and *spike-NSP3* gene segments (Figure 4). For example, every FCoV gene sequence for the membrane gene from FIP cases fell within a major cluster consisting of 3 principal clades (Figure 4). By contrast, 127/154 (82%) virus gene sequences from FECV-asymptomatic cats sorted in 2 separate clades that were distinct (100 bootstrap statistical support) from the viral gene sequences of FIP cases (Figure 4). Similar reciprocal monophyly of 140 *NSP7b* sequences was obtained for FIP cases versus FECV-asymptomatic cats (Figure 4). A consistent disease driven phylogeographic sorting was also observed for the 1,017-bp sequence spanning the *spike-NSP3* genes, albeit with less statistical resolution, likely because of evolutionary constraints on gene divergence in this region (Figure 4). Together the remarkable reciprocal monophyly in these 3 genes supports the predictions of the circulating virulent-avirulent strain hypothesis illustrated in Figure 1.

Samples from 1 cat, Fca-4590, were particularly informative. The virus was isolated from the cat predisease, and then again 7 months later postdisease. Fca-4590 was asymptomatic but infected with FECV in May 2004. FCoV sampling from that month showed strong (high bootstrap) affiliation with the FECV-asymptomatic clades for the membrane and the *NSP7b* genes. However, virus isolated

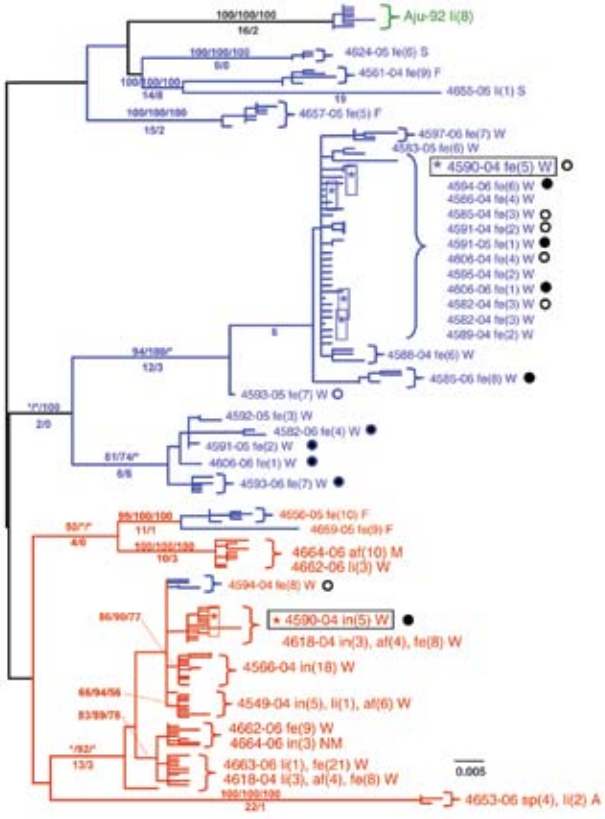


Figure 4. Maximum-likelihood (ML) phylogenetic tree of unique sequences from 3 feline coronavirus (FCoV) genes, *membrane*, *NSP 7b*, and *spike-NPS3* (see Figure 3), showing monophyly correlating to disease status. Cloned sequences from feline infectious peritonitis (FIP) cases are shown in red, feline enteric coronavirus (FECV) asymptomatic cats are shown in blue, and FCoV virulent strain from Aju-92 (cheetah) is in green. Shown above are *membrane* 575-bp sequences (ML –ln L = 3086.20787 best tree found by maximum parsimony [MP] tree: length = 493, CI = 0.551724, RI = 0.0926505); additional sequences are shown in an online expanded version of this figure, available from www.cdc.gov/EID/content/15/9/1445-F4.htm. The number of FIP cases and FECV-asymptomatic cats and number of cloned sequences is indicated in parenthesis. Each sequence is labeled as follows: source farm (W, Weller Farm; F, Frederick Animal Shelter; S, Seymour Farm; M, Mount Airy Shelter; A, Ambrose Farm), 4-digit cat identification number, tissue source (fe, feces; af, ascites fluid; co, colon; li, liver; sp, spleen; in, intestine; je, jejunum; ln, lymph node), 2-digit year (eg., 04 = 2004), and number of clones for each sequence. Bootstrap values are shown (MP/minimum evolution/ML) above branches. Where ML tree was congruent with MP tree, branch lengths are indicated below branches; the number of homoplasies is in parenthesis after the branch length. Number of cats and number of clones assessed are listed in Figure 3, panel B. Virus sequence obtained from cat no. 4590 in May 2004 and at the time of death due to FIP in December 2004 is indicated by box. The 4590-transitional individual serial samples are indicated with open circles (first sample) and solid circles (second sample). Scale bar indicates substitutions/site.

7 months later in December 2004 after FIP developed in Fca-4590 fell within the FIP-case clades (also with high bootstrap), and was indistinguishable from FCoV isolated from other cats with FIP. This finding suggested that the pathogenic FIP-case type of FCoV infected this cat subsequent to its infection with an avirulent FECV and apparently replaced it.

Tissue-specific differentiation within each cat was minimal (Figure 4). By contrast, there were notable locale-specific distinctions within the sick and healthy cats (Figure 4). For example, the FECV strains in asymptomatic cats from the Weller household were associated together in a major FECV subclade; strains in cats from the Frederick Animal Shelter were classified in a different subclade, nested within the FECV-asymptomatic clade (Figure 4). The archival FCoV virulent strain (Aju-92), isolated from cheetahs in Oregon in 1982 (*30*), defined a phylogenetic lineage distinctive from the FIP and FECV-asymptomatic clades resolved in the Maryland domestic cats (Figure 4).

Nucleotide sequences of *membrane* and *NSP 7b* generated in this study were translated to amino acid sequences (online Appendix Figure 1, available from www.cdc.gov/EID/content/15/9/1445-appF1.htm). Relative to pathogenesis, 5 informative amino acid sites were found in the membrane protein at positions 108, 120, 138, 163, and 199 (based on reference sequence for TGEV GenBank no. NP058427) (*22*), giving rise to 6 composite genotypes potentially diagnostic of FIP cases versus FECV-asymptomatic cats (online Appendix Table 2). Among the 8 cats with FIP, 19 FECV-asymptomatic cats, and 1 cheetah with FIP, 6 composite genotypes were identified based on these 5 diagnostic sites (online Appendix Table 2).

All domestic cats with FIP diagnosed by pathologic or immunohistochemical changes displayed the amino acid signature of either YIVAL (I) or YIIAL (II); infected cats without clinical FIP had the HIIVI (III), HIIVL (IV), HVIAL (V), YVVAL (VI), or YIVAL (I) haplotype. No FIP cases had haplotype III, IV, V, or VI, whereas 3 FECV-asymptomatic cats carried the YIVAL signature found predominately in FIP cases (Fca-4594, 4624, and 4657; online Appendix Table 2). Of these, 2 cats (4624 and 4657) were euthanized at the time of sampling (all euthanized FECV-asymptomatic cats are highlighted in light green in online Appendix Table 2); therefore, whether clinical FIP would have later developed in these cats is unknown. The other exception, cat 4594, was sampled twice (in 2004 and again in 2006); the switch in genetic signature from YIVAL in 2004 to HIIVI in 2006 may indicate that this cat was able to clear a virulent FIPV strain after the 2004 sampling and become reinfected with an avirulent strain by 2006. Although a strong phylogenetic signal differentiating FIP cases from FECV-asymptomatic cats was seen in *NSP 7b* (Figure 4), no diagnostic amino acid changes specific to FIP cases vs. FECV-asymptomatic controls were found in the *NSP 7b* nucleotide or amino acid alignments. In contrast to the monophyletic findings in the *membrane, NSP 7b,* and *spike-NSP3* genes, cloned viral sequences of *pol 1a,* were paraphyletic in terms of disease phenotype (online Appendix Figure 2, available from www.cdc.gov/EID/content/15/9/1445-appF2.htm).

Discussion

Infection with FCoV is common in cats throughout the world, although in most cats the virus causes no clinical signs. However, in some cats, FCoV infection is associated with the development of the progressive and fatal disease manifestation of FIP. This disease is among the most serious viral infections in cats, not only because of its fatal nature, but also because of the difficulties in diagnosing FIP antemortem and controlling the spread of FCoV. We have presented a molecular virologic study of naturally occurring FCoV infection and phylogenetic analysis of the cloned virus sequences obtained from the *membrane, NSP 7b, spike-NSP3,* and *pol 1a* genes isolated from domestic cats located in Maryland households infected with FCoV during 2004–2006. We observed predominately monophyletic clustering of strains correlating with disease phenotype in *membrane and NSP 7b* genes consistent with the circulating virulent/avirulent strain hypothesis of FIP pathogenesis, which calls into question the in vivo mutation hypothesis.

The amino acid alignments presented in online Appendix Figure 1 clearly demonstrate that in the FIPV cases included in this study the genotypes correlated with disease phenotype are ancestrally derived and not the result of a few de novo mutations. Given the clear genetic differentiation between viruses from FIP cases and FECV asymptomatic cats in multiple gene segments, we infer that cats become reinfected with new strains of FCoV from external sources, rather than by in vivo mutations. Cats in our study were not co-infected with multiple strains of FECV and FIPV at the same time and were generally infected with one predominant virus strain. Two exceptions to this finding in our study were cats with cases of FIPV (Fca-4662 and 4664) that from which distinct gastrointestinal (feces or intestine) and systemic (liver and/or ascites fluid) viral isolates were obtained, which indicates that in vivo superinfection does occur (Figure 4; online Appendix Table 2).

A role of the membrane protein in FIP pathogenesis seems likely, given its known functions in other coronaviruses. The membrane protein is the most abundant structural protein with important functions in virus budding and with cell-mediated host immunity (*31*). The specific functions of the membrane protein amino acid sequences have been determined in severe acute respiratory syndrome (SARS)–CoV (*32*). Aligning the sequences from this study with SARS-CoV, the first diagnostic amino acid site 108

aligns to a site just upstream from the second transmembrane helix (online Appendix Figure 1). A tyrosine at position 108, which is found in all FIPV biotypes and shared among SARS-CoV, has a neutral polarity (in contrast to a histidine there, found in most FECV biotypes, which have a positive polarity) and may play a role in the stability of the virus within the membrane. Site 120 aligns within the third transmembrane helix, site 138 aligns just downstream to the transmembrane helice, site 163 aligns within the C-terminus, which projects inside the virus particle, and site 199, also within the C-terminus domain, aligns within a defined SARS-immunodominant epitope (*32*) (Figure 5).

The demonstration of 6 naturally occurring composite genotypes based on 5 variable sites in the membrane protein amino acid alignment that are highly correlative with disease phenotype (online Appendix Table 2) offers specific opportunities for developing diagnostics and for the preventive management of this disease. By extending this study to additional cat populations in disparate geographic locations, designing chimeric FCoV challenge experiments, and investigating host genetic correlations with pathogenesis, we will be able to further discern the causative factors in FIP pathogenesis. Fca-4594, which was infected with the disease-associated genotype composite without succumbing to FIP, suggests additional requirements for viral pathogenesis. As has been suggested in the outbreak of FIP in a colony of captive cheetahs (*33*), host immune genetics may play a role.

Both the viral strain and host immune genes contribute to disease progression and virus-related death, such as AIDS progression in HIV infection. With the recent publication of the full cat genome sequence (*34*) and the viral genotype composites described here, new genomic tools are now available to proceed with both viral and host genetic association studies in the pathogenesis in FCoV infection, a model for coronavirus infection in humans, such as SARS-CoV.

Acknowledgments

We thank Linda Rawls, Beth Weller, and Frederick County Animal Shelter for help with sample collection; Bailey Kessing and Nicole Crumpler for sample database management; Dahlem Smith for histopathologic evaluation; and James Evermann for immunohistochemical assistance.

This study was supported in part by the National Cancer Institute, National Institutes of Health, under contract N01-CO-12400; and the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

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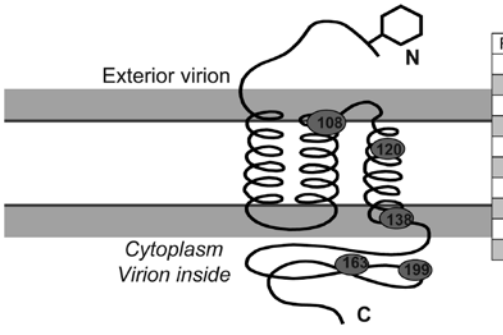
References

1. Addie DD. Clustering of feline coronaviruses in multicat households. Vet J. 2000;159:8–9. DOI: 10.1053/tvjl.1999.0429
2. Addie DD, Jarrett O. A study of naturally occurring feline coronavirus infections in kittens. Vet Rec. 1992;130:133–7.
3. Kennedy M, Citino S, McNabb AH, Moffatt AS, Gertz K, Kania S. Detection of feline coronavirus in captive *Felidae* in the USA. J Vet Diagn Invest. 2002;14:520–2.
4. Pedersen NC. A review of feline infectious peritonitis virus infection: 1963–2008. J Feline Med Surg. 2009;11:225–58. DOI: 10.1016/j.jfms.2008.09.008
5. Foley JE, Poland A, Carlson J, Pedersen NC. Risk factors for feline infectious peritonitis among cats in multiple-cat environments with endemic feline enteric coronavirus. J Am Vet Med Assoc. 1997;210:1313–8.
6. Pedersen NC, Evermann JF, McKeirnan AJ, Ott RL. Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. Am J Vet Res. 1984;45:2580–5.
7. de Groot RJ. Feline infectous peritonitis. In: Siddell SG, editor. The *Coronoviridae*. New York: Plenum Press; 1995. p. 293–309.
8. Weiss RC, Scott FW. Pathogenesis of feline infectious peritonitis: nature and development of viremia. Am J Vet Res. 1981;42:382–90.
9. Kipar A, Kohler K, Leukert W, Reinacher M. A comparison of lymphatic tissues from cats with spontaneous feline infectious peritonitis (FIP), cats with FIP virus infection but no FIP, and cats with no infection. J Comp Pathol. 2001;125:182–91. DOI: 10.1053/jcpa.2001.0501
10. Kipar A, Meli ML, Failing K, Euler T, Gomes-Keller MA, Schwartz D, et al. Natural feline coronavirus infection: differences in cytokine patterns in association with the outcome of infection. Vet Immunol Immunopathol. 2006;112:141–55. DOI: 10.1016/j.vetimm.2006.02.004



Residue	Symbol	Amino acid	Polarity	Hydro-
108	H	Histidine	Positive	philic
108	Y	Tyrosine	Neutral	philic
120	V	Valine	Nonpolar	phobic
120	I	Isoleucine	Nonpolar	phobic
138	I	Isoleucine	Nonpolar	phobic
138	V	Valine	Nonpolar	phobic
163	V	Valine	Nonpolar	phobic
163	A	Alanine	Nonpolar	phobic
199	I	Isoleucine	Nonpolar	phobic
199	L	Leucine	Nonpolar	phobic

Figure 5. Diagram of membrane protein containing 3 transmembrane helices, an external N terminus and an internal carboxy terminus. Approximate position of 5 variable diagnostic amino acid sites (see Table 2) as determined by sequence comparison to severe acute respiratory syndrome coronavirus (*32*). Amino acid residue, polarity, and hydrophobicity or hydropholicity is stated.

11. Hunziker L, Recher M, Macpherson AJ, Ciurea A, Freigang S, Hengartner H, et al. Hypergammaglobulinemia and autoantibody induction mechanisms in viral infections. Nat Immunol. 2003;4:343–9. DOI: 10.1038/ni911
12. Poland AM, Vennema H, Foley JE, Pedersen NC. Two related strains of feline infectious peritonitis virus isolated from immunocompromised cats infected with a feline enteric coronavirus. J Clin Microbiol. 1996;34:3180–4.
13. Vennema H, Poland A, Foley J, Pedersen NC. Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. Virology. 1998;243:150–7. DOI: 10.1006/viro.1998.9045
14. Rottier PJ, Nakamura K, Schellen P, Volders H, Haijema BJ. Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. J Virol. 2005;79:14122–30. DOI: 10.1128/JVI.79.22.14122-14130.2005
15. Stoddart CA, Scott FW. Intrinsic resistance of feline peritoneal macrophages to coronavirus infection correlates with in vivo virulence. J Virol. 1989;63:436–40.
16. Haijema BJ, Volders H, Rottier PJ. Switching species tropism: an effective way to manipulate the feline coronavirus genome. J Virol. 2003;77:4528–38. DOI: 10.1128/JVI.77.8.4528-4538.2003
17. Can-Sahna K, Soydal Ataseven V, Pinar D, Oguzoglu TC. The detection of feline coronaviruses in blood samples from cats by mRNA RT-PCR. J Feline Med Surg. 2007;9:369–72. DOI: 10.1016/j.jfms.2007.03.002
18. Dye C, Siddell SG. Genomic RNA sequence of feline coronavirus strain FCoV C1Je. J Feline Med Surg. 2007;9:202–13. DOI: 10.1016/j.jfms.2006.12.002
19. Hartley O, Klasse PJ, Sattentau QJ, Moore JP. V3: HIV's switch-hitter. AIDS Res Hum Retroviruses. 2005;21:171–89. DOI: 10.1089/aid.2005.21.171
20. Ballesteros ML, Sanchez CM, Enjuanes L. Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. Virology. 1997;227:378–88. DOI: 10.1006/viro.1996.8344
21. Saif LJaS. K. Transmissible gastroenteritis virus and porcine respiratory coronavirus. In: Zimmerman JJ, editor. Diseases of swine. 9th ed. Ames (IA): Iowa State University Press; 2006. p. 489–516.
22. Sanchez CM, Izeta A, Sanchez-Morgado JM, Alonso S, Sola I, Balasch M, et al. Targeted recombination demonstrates that the spike gene of transmissible gastroenteritis coronavirus is a determinant of its enteric tropism and virulence. J Virol. 1999;73:7607–18.
23. Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, et al. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med. 2003;9:921–7. DOI: 10.1038/nm887
24. Anishchenko M, Bowen RA, Paessler S, Austgen L, Greene IP, Weaver SC. Venezuelan encephalitis emergence mediated by a phylogenetically predicted viral mutation. Proc Natl Acad Sci U S A. 2006;103:4994–9. DOI: 10.1073/pnas.0509961103
25. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25:4876–82. DOI: 10.1093/nar/25.24.4876
26. Maddison DRaM. W.P. MacClade 3.05. Sunderland (MA): Sinauer; 1995.
27. Swofford DL. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Sunderland (MA): Sinauer; 2002.
28. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics. 1998;14:817–8. DOI: 10.1093/bioinformatics/14.9.817
29. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform. 2004;5:150–63. DOI: 10.1093/bib/5.2.150
30. Pearks Wilkerson AJ, Teeling EC, Troyer JL, Bar-Gal GK, Roelke M, Marker L, et al. Coronavirus outbreak in cheetahs: lessons for SARS. Curr Biol. 2004;14:R227–8. DOI: 10.1016/j.cub.2004.02.051
31. Rottier PJ. The coronavirus membrane glycoprotein. In: Siddell SG, editor. The *Coronaviridae*. New York: Plenum Press; 1995. p. 115–40.
32. He Y, Zhou Y, Siddiqui P, Niu J, Jiang S. Identification of immunodominant epitopes on the membrane protein of the severe acute respiratory syndrome-associated coronavirus. J Clin Microbiol. 2005;43:3718–26. DOI: 10.1128/JCM.43.8.3718-3726.2005
33. Heeney JL, Evermann JF, McKeirnan AJ, Marker-Kraus L, Roelke ME, Bush M, et al. Prevalence and implications of feline coronavirus infections of captive and free-ranging cheetahs (*Acinonyx jubatus*). J Virol. 1990;64:1964–72.
34. Pontius JU, Mullikin JC, Smith DR, Lindblad-Toh K, Gnerre S, Clamp M, et al. Initial sequence and comparative analysis of the cat genome. Genome Res. 2007;17:1675–89. DOI: 10.1101/gr.6380007

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etymologia

Borna disease virus

[bor′nə]

Borna disease virus was named after the town of Borna in Saxony, southeastern Germany, where in 1885 many horses in a German cavalry regiment died of a fatal neurologic disease. The ill horses exhibited abnormal behavior—running about excitedly, walking into walls, being unable to chew food. A similar disease had been observed in horses, sheep, and cattle for more than 100 years. The causative agent was later found to be a negative-stranded RNA virus, which may also be a human pathogen.

Source: Carabone KM. Borna disease virus and its role in neurobehavioral disease. Washington: ASM Press; 2002.

Avian Bornaviruses in Psittacine Birds from Europe and Australia with Proventricular Dilatation Disease

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To determine whether avian bornaviruses (ABVs) were a factor in proventricular dilatation disease (PDD), we used immunohistochemistry, reverse transcription–PCR, and nucleotide sequence analysis to examine paraffin wax–embedded or frozen tissue samples of 31 psittacine birds with this disease. PDD is a fatal disease of psittacine birds associated with nonsuppurative encephalitis and ganglioneuritis of the upper intestinal tract. Tissue samples had been collected from 1999 through 2008 in Austria, Switzerland, Hungary, and Australia. Immunohistochemical demonstration of viral antigen within the brain and vegetative nerve system of the gastrointestinal tract provides strong evidence for a causative role of ABVs in this condition. Partial sequences of nucleoprotein (p40) and matrix protein (gp18) genes showed that virus in most of our cases belonged to the ABV-2 and ABV-4 groups among the 5 genogroups described so far. Viral sequences of 2 birds did not match any of the described sequences and clustered together in a new branch termed ABV-6.

Proventricular dilatation disease (PDD), a serious and frequent disease of predominantly psittacine birds, was reported for the first time in the late 1970s and early 1980s in the United States, in Germany, and in Switzerland (*1*,*2*). PDD affects >50 species of the order Psittaciformes, and single cases of a clinically and morphologically largely identical disease have also been found in nonpsittacine species (*3*,*4*). The disease is characterized by nonsuppurative ganglioneuritis of the vegetative nerve plexuses of the crop, proventriculus, gizzard, and duodenum. In most cases, PDD is accompanied by nonsuppurative encephalomyelitis, and inflammation has also been noted in the peripheral nerves (*5*), myocardium (*1*,*6*,*7*), and adrenal glands (*7*).

Although a possible viral cause has been considered since the disease was recognized (*1*,*2*), and electron microscopic examination of affected tissues showed structures suggestive of viral particles, no convincing evidence for infection with a specific virus has been produced in the past 30 years. Recently, however, Kistler et al. (*8*) and Honkavuori et al. (*9*) provided evidence that PDD is associated with the presence of novel virus species within the family *Bornaviridae*, provisionally termed avian bornavirus (ABV). Using advanced molecular genetic technologies, they found sequences of at least 5 genetic subgroups of ABV in clinical specimens from most birds with PDD, but in none of the controls. These outstanding pioneer achievements have opened the doors to further research into the epizootiology, pathogenesis, and prevention of the disease.

In this study, we found ABVs within tissue lesions consistent with PDD by using immunohistochemical (IHC) testing, and we examined the distribution pattern of the ABV genotypes in various parts of the world. In addition, we describe tools for detecting viral signatures in archived paraffin wax–embedded tissue samples that are useful for retrospective studies.

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DOI: 10.3201/eid1509.090353

Methods

Tissue Samples

For this study, we had access to paraffin wax–embedded tissue samples from 25 psittacine birds (20 species) from Austria (N = 13), Switzerland (N = 11), and Australia (N = 1), which were obtained for pathologic examination from 1999 through 2007 (online Appendix Table, available from www.cdc.gov/EID/content/15/9/1453-appT.htm). Each of these birds had received a diagnosis of PDD from the results of clinical, pathoanatomical, or pathohistologic examination. Samples from the brain and, if available, from the crop, proventriculus, and gizzard of all birds (except the bird from Australia) were used for IHC analysis and reverse transcription–PCR (RT-PCR). From the 4 birds with the most abundant immunostaining in these organs, other tissues, such as heart (n = 3), lung (n = 4), liver (n = 3), kidney (n = 3), or skin (n = 2) also underwent IHC testing.

In addition, frozen samples of the brain and proventriculus of 6 psittacine birds from Switzerland (n = 4) and Hungary (n = 2), also with clinical, pathoanatomical, and pathohistologic diagnosis of PDD, were used for investigation by RT-PCR only. Paraffin wax–embedded tissue samples of brain from 5 psittacine birds with diagnoses other than PDD were used as negative controls.

IHC Testing

Sections (4 μm) were cut from tissue blocks, placed on positively charged glass slides (Menzel Gläser, Braunschweig, Germany), and subjected to staining by IHC with 3 anti–Borna disease virus (BDV) antibodies: 2 monoclonal antibodies directed against the N protein of BDV (Bo18: provided by S. Herzog, 38/17C1: provided by L. Stitz), and 1 polyclonal antibody, directed against the phosphoprotein of BDV (provided by W.I. Lipkin). Selected slides of brain tissue were also stained with a polyclonal antibody for identification of astrocytes (anti–glial fibrillary acidic protein [GFAP], Dako, Glostrup, Denmark). After deparaffination and rehydration, antigen retrieval (except for GFAP) was performed by heating the slides in citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. Sections were then incubated with 1:10 diluted normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 60 min, directly followed by an overnight incubation with the antibodies mentioned above at 4°C (dilutions: Bo 18: 1:2,000; 38/17C1 and antiphoshoprotein: 1:2,500, anti-GFAP: 1:1,000). After being washed in phosphate-buffered saline, the tissues were incubated with biotinylated antimouse or antirabbit immunoglobulin G (Vector Laboratories, dilution 1:400) for 30 min, followed by staining using the Vectastain ABC Kit (Vector Laboratories) for 60 min. The reaction was visualized by using a 3,3′ diaminobenzidine Substrate Kit for Peroxidase (Vector Laboratories). After counterstaining with hemalum and dehydrating were carried out, the slides were placed under coverslips with Neomount (VWR, Vienna, Austria). Brain sections of a BDV-infected horse and a psittacine bird with PDD in which the diagnosis ABV infection had been proven by sequencing of an RT-PCR amplification product were used as positive controls. Brain sections of psittacines with diagnoses other than PDD were used as negative controls.

Nucleic Acid Extraction and RT-PCR

Viral RNA was extracted from 10-μm sections of paraffin wax–embedded psittacine tissue samples. Pools of 3 to 5 sections of each block were processed. The origin of the samples is indicated in the online Appendix Table. The tissue sections were deparaffinized by incubation with 1 mL xylene for 20 min at 37°C, followed by pelleting the tissues by centrifugation at 16,000 × *g* for 5 min at room temperature. Xylene was removed, and the pellets were resuspended in 1 mL RNase-free ethanol for 5 min at room temperature. The samples were centrifuged again at 16,000 × *g* for 5 min at room temperature, and the ethanol treatment was repeated. After centrifugation, the ethanol was removed, and the pellets were air-dried. Thereafter, the tissue samples were resuspended in 250 μL ATL tissue lysis buffer (QIAGEN, Hilden, Germany) and 25 μL Proteinase K (QIAGEN) was added. Samples were digested with proteinase for 16 h at 55°C, followed by an enzyme-inactivation step for 8 min at 95°C. Viral RNA was extracted from 140 μL of the tissue lysates by using the QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's recommendations.

BDV-specific nucleic acid sequences deposited in GenBank database, including the 5 ABV genotypes described to date (*8*), were aligned and analyzed for conserved genomic sections. Bornavirus-specific universal oligonucleotide primer pairs were designed, which annealed to putative N protein (forward primer 5′-CATGAGGCTATWGATTGGATTA-3′ and reverse primer 5′-TAGCCNGCCMKTGTWGGRTTYT-3′) and to matrix (M) protein gene regions (forward primer 5′-CAAGGTAATYGTYCCTGGATGG-3′ and reverse primer 5′-ACCAATGTTCCGAAGMCGAWAY-3′) of ABVs, respectively. These primers corresponded to nt positions 632–653 and 999–1020 (N gene) and 1908–1929 and 2238–2259 (M gene) of the complete genome of ABV strain "bil," GenBank accession no. EU781967 (*8*). Because mostly paraffin wax–embedded tissue samples were used as sample material, primers for the amplification of relatively short PCR products were designed (389 and 352 bp, respectively), to reduce the chance of false-negative reactions due to the RNA fragmentation effect of the formaldehyde fixation.

ABV RNAs were reverse-transcribed and amplified with a continuous RT-PCR method by using a One Step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. Primers were used at final concentrations of 0.8 µmol/L. Amplifications were performed in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). The temperature profile for the RT-PCR was as follows: 30 min at 50°C, 15 min at 95°C, 45× (30 s at 94°C, 30 s at 50°C, and 30 s at 72°C), and 7 min at 72°C. RNA extracts from psittacine organs without indication of PDD served as negative controls. PCR products were subjected to electrophoresis in 1.5% Tris acetate–EDTA agarose gels and stained with ethidium bromide.

Sequencing and Sequence Analysis

PCR products were purified with the Quantum Prep PCR Kleen Spin Columns (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Fluorescence-based direct sequencing of the amplicons was performed in both directions by using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) (*13*). Nucleotide sequences were identified by the Basic Local Alignment Search Tool (BLAST [*14*]) and were aligned by using the Align Plus program, version 4.1 (Scientific and Educational Software, Cary, NC, USA). Multiple alignments for phylogenetic analyses were created by using the ClustalX program (*15*). Phylogenetic analyses were conducted by the neighbor-joining algorithm. Bootstrap resampling analyses of the phylogenetic trees were performed on 1,000 replicates. Trees were drawn with the help of the TreeView 1.6.6 software (Scientific and Educational Software). Besides the nucleotide sequences obtained in this study, all ABV sequences of the investigated genomic regions, which had been deposited in the GenBank database by other authors (*8*,*9*), were also included in the sequence alignments and phylogenetic analyses. The ABV sequences described in this article were submitted to GenBank database under accession nos. FJ794724–FJ794754 (online Appendix Table).

Results

IHC Testing

The monoclonal antibodies used (Bo18, 38/17C1), which produced clear specific immunoreactivity in the BDV-infected equine brain control section, showed negative results on the avian control brain and tissues of PDD birds. The polyclonal antibody directed against recombinant BDV phosphoprotein, however, showed positive results (Figures 1, 2). All PDD samples examined (N = 24) showed immunoreactivity in at least 1 brain sample. Other positive tissues were the proventriculus, gizzard, crop, small intestine, heart, and lung. In the brain, positive results were randomly distributed, without predilection to specific neuroanatomic locations. The quantity of positive cells ranged from single cells in the entire section to large numbers of positive cells, which in certain brain areas accounted for up to one third of all cells. The positive cells were found more frequently in the gray matter, but were also found in the white matter. Neurons were consistently found to be positive (Figure 1, panels C–F). Their staining pattern was variable and included positive nuclei and negative cytoplasm, positive nuclei and cytoplasm, or negative nuclei and positive cytoplasm.

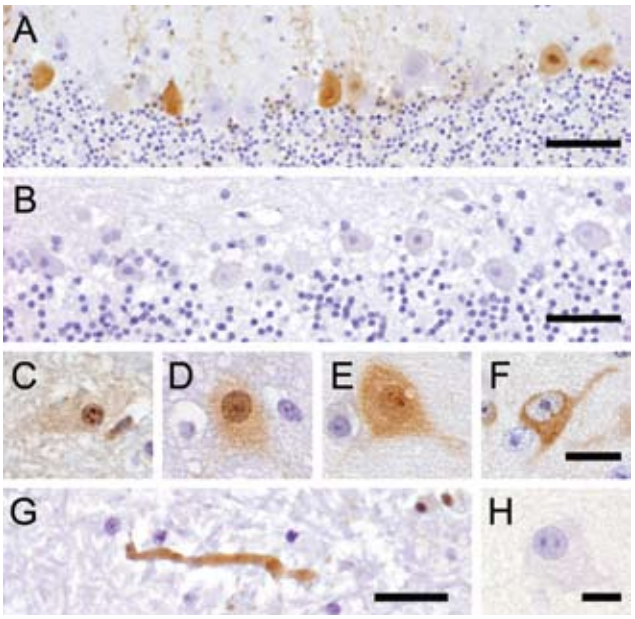

Figure 1. Avian bornavirus protein demonstrated by immunohistochemical testing in the central nervous system of birds with proventricular dilatation disease (PDD). A) within nuclei, cytoplasm and dendrites of several Purkinje cells of the cerebellum, bar = 50 µm; B) negative control: no immunoreactivity of Purkinje cells in a PDD-negative bird, bar = 50 µm; C–F), different phenotypes of positive neurons: C) within neurons, viral protein is expressed within intranuclear inclusion bodies; D) diffusely within the nucleus accompanied by faint cytoplasmic staining; E) both, within the nucleus and cytoplasm, with more intense staining of intranuclear inclusion bodies; F) exclusively within the cytoplasm and the nucleus spared, bar = 12.5 µm; G) within an axon in the white matter of the medulla oblongata, bar = 25 µm; H, negative control: no immunoreactivity of a cerebral neuron in a PDD-negative bird, bar = 12.5 µm.

In several cases, a peculiar nuclear staining pattern occurred, with 1 or several distinctly stained spheroid intranuclear bodies, either present as a sole nuclear immunoreaction or as a reaction in context with diffuse nuclear or cytoplasmic staining, or as both. Occasionally, neuronal processes in gray and white matter were distinctly labeled (Figure 1, panel G). In addition, astrocytes occasionally

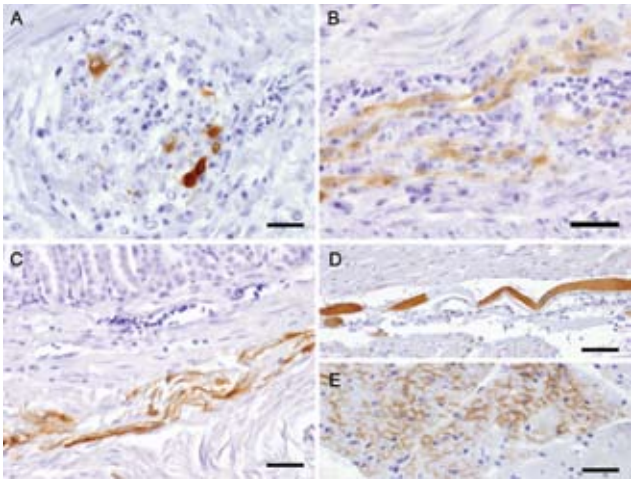

Figure 2. Avian bornavirus protein demonstrated by immunohistochemical testing in extracerebral locations. A) within neurons of proventricular intramural vegetative ganglia with inflammatory infiltration, bar = 25 µm; B) within nerve fibers of the myenteric plexus of gizzard, bar = 25 µm; C) within smooth muscle fibers of the proventricular wall, bar = 50 µm; D) within a modified muscle fiber of the conductive system of the heart, bar = 50 µm; E) within myocardiocytes, bar = 25 µm.

showed positive results, with several specifically stained intranuclear bodies. Identity of astrocytes was confirmed by IHC demonstration of GFAP in the cytoplasm of the same cells in a directly adjacent serial section. Some areas also displayed more or less intense immunoreactivity of the neuropil. There was no immunostaining in the negative controls (Figure 1, panels B, H). Most samples (n = 16) showed mild-to-severe nonsuppurative inflammation in the brain sections examined, but several samples (n = 8) did not show inflammatory infiltrates. No direct correlation was found between location of inflammation and presence of viral antigen. In the proventriculus, crop, gizzard, and small intestine, intramural or subserous nerve plexus and ganglia had positive results. Nuclei and cell bodies of enteral ganglia as well as nerve fibers had distinctly positive results (Figure 2, panels A, B). All examined enteric vegetative ganglia or nerves showed mild-to-severe inflammatory infiltration. In some cases, smooth muscle fibers also showed strong immunoreactivity (Figure 2, panel C). In the heart, vegetative nerve fibers, large modified muscle fibers of the conduction system (Figure 2, panel D), and foci of normal heart muscle fibers (Figure 2, panel E) showed positive results. In the lung, some interstitial nerve fibers also showed positive results. No unequivocal identification of positive staining was found in other organs, such as liver, kidney, or skin.

RT-PCR and Nucleotide Sequence Analysis

Amplification products of previously calculated sizes were generated by the RT-PCR assays. No amplification products were obtained from PDD-negative psittacine samples. In several cases, brain and proventriculus from the same animal were simultaneously tested. In general, brain samples gave more frequent and more intensive results; however, in some cases, proventriculi showed positive results, and brains showed negative results. The results of IHC and RT-PCR investigations were mainly consistent. However, in the case of 2 samples from Austria with IHC-positive results, the results of repeated RT-PCRs were always negative (samples 801–01 and 1688–04; online Appendix Table). All samples were tested with N–and M gene–specific primer pairs. The M protein gene regions of all 29 samples were successfully amplified, but at the N coding region, only 22 samples showed unambiguously positive reactions. The nucleotide sequences of the amplification products were identified in at least 2 independent sequencing reactions. In several electrophorograms, overlapping peaks were detected at certain loci, even after repeated processing (repeated nucleic acid extraction, RT-PCR, and sequencing) of the samples. These variable loci were often in common in different samples, and in a few cases, unambiguous consensus sequences could not be obtained because of this fact.

Amplification products from brain and proventriculus of the same birds were sequenced in 12 samples. Notably, in 2 samples, nucleotide sequences obtained from brain and proventriculus of the same parrot differed in the N coding region by a few nucleotides, although they were identical in the M gene region. In all other cases, however, the sequences obtained from brain and proventriculus of the same birds were identical. The partial N gene sequence (between nt positions 654 and 998, referred to the complete ABV genome record EU781967) of 22 samples, and the partial M gene sequence (between nt positions 1930 and 2237) of 29 samples were determined. The sequences shared the highest identity rates with ABV sequences after BLAST search against the GenBank database. The nucleotide identity rates of the newly determined and the GenBank sequences varied between 71% and 100% in the nucleoprotein gene region, and between 68% and 100% in the M protein gene region. The sequences from 14 samples showed the highest similarity rates (92% to 100%) to ABV-2 sequences, and 13 samples were most similar (94% to 100%) to ABV-4 sequences. The M gene sequences of the samples 281–01 and H03–2080, however, were only 71% to 82% identical to any other known bornavirus sequence. From these samples, amplification products were not obtained with the N gene–specific primer pair.

Phylogenetic trees (Figures 3, 4), based on the N and M protein gene regions, showed similar structures. The sequences did not exhibit any clustering according to either the collection year, country of origin, or host species. ABV sequences from different countries in Europe and from

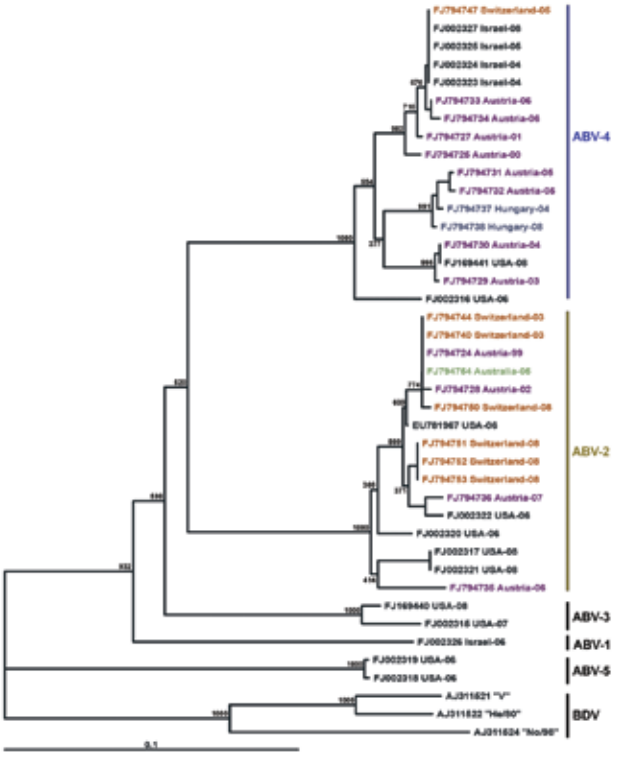

Figure 3. Phylogram illustrating the genetic relationship among avian bornavirus (ABV) genotypes, based on a partial nucleoprotein gene region. Three representatives of Borna disease virus (BDV) were used as outgroups. Scale bar indicates genetic distance; the bootstrap support values are shown for the main nodes. ABVs are identified by GenBank accession number/country of origin/year of collection. Further details are shown in the online Appendix Table (available from www.cdc.gov/EID/content/15/9/1453-appT.htm). Nucleotide sequences determined in this study are highlighted in different colors according to their country of origin. The main ABV genogroups are indicated.

Australia were similar, or even homologous to each other. Moreover, the newly determined sequences proved to be frequently similar or homologous to GenBank sequences derived from samples collected in the United States and in Israel. The newly identified sequences clustered within the same main branches in the N and M gene trees. Two similar M protein gene sequences (281–01 and H03–2080), however, exhibited low identity rates to all other sequences and formed a distinct branch in the phylogenetic tree, which was clearly separated from all other groups of ABVs and mammalian bornaviruses. Consequently, we suggest that this unique cluster be accepted as a novel ABV genotype, designated ABV-6. The bootstrap analysis supported the main clustering of the consensus trees.

Discussion

The successful IHC demonstration of ABV antigens in birds with PDD by using a polyclonal serum against the phosphoprotein of BDV shows that, despite their diversity, ABVs and BDV share epitopes. Positive results for virus were found for neurons and nerve fibers of the brain, and in ganglia, nerve fibers, and smooth muscle fibers of the upper digestive tract as well as in myocytes and fibers of the conductive system of the heart.

Typical for the presence of bornaviruses is the location of viral protein within distinct intranuclear inclusions (called Joest-Degen inclusion bodies in classic BD of neurons, which have been shown to be sites of viral transcription and translation (*16*,*17*). In the PDD samples analyzed here, such immunoreactive intranuclear bodies were consistently found in brain neurons. In at least 2 studies, histologic pictures of celiac ganglia undoubtedly show intranuclear inclusion bodies, which strongly resemble the intranuclear inclusion bodies known from BD (*2*,*18*). PDD is characterized by an immunologic attack of infiltrating immune cells on the autonomous nervous system of the upper digestive tract. In classic BD, however, this phenomenon is not observed. Here, inflammation remains confined to the central

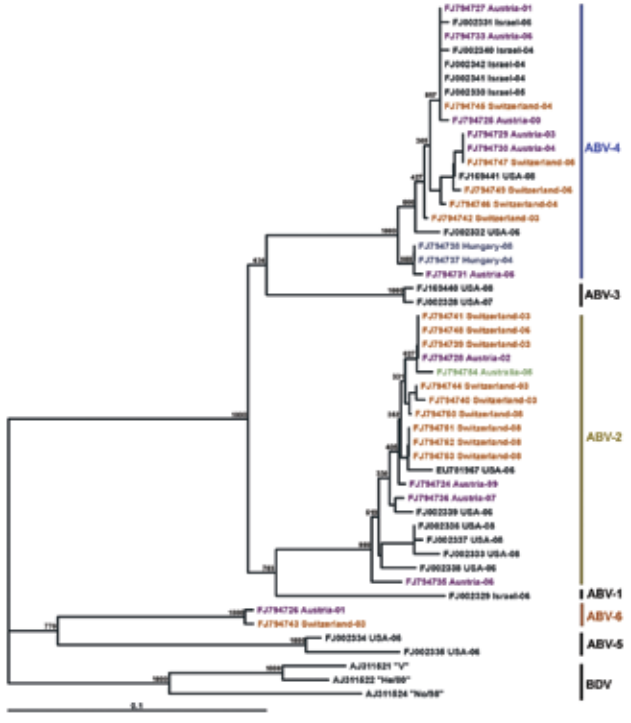

Figure 4. Phylogram illustrating the genetic relationship among avian bornavirus (ABV) genotypes, based on a partial matrix protein gene region. Three representatives of BDV were used as outgroups. Scale bar indicates genetic distance; the bootstrap support values are shown for the main nodes. ABVs are identified by GenBank accession number/country of origin/year of collection. Further details are shown in the online Appendix Table (available from www.cdc.gov/EID/content/15/9/1453-appT.htm). Nucleotide sequences determined in this study are highlighted in different colors according to their country of origin. The main ABV genogroups are indicated.

nervous system, although centrifugal spread of virus into peripheral nerves and autonomic nerve fibers and ganglia has been shown in experimentally infected rats (*19*,*20*).

Some of the birds showed viral antigen in extraneural tissues, such as smooth or heart muscle fibers. These findings are comparable to the situation in rats that were experimentally infected as newborns, which are immunotolerant to the virus, or in animals in which artificial immunosuppression is induced. In the absence of an efficient immune response, these animals show that spread of infectious virus to several nonneural tissue cells, such as hepatocytes, tubular epithelial cells of the kidney, and myocytes of the intestine and heart (*19*,*21*,*22*). Whether birds with PDD have deficient humoral or cellular immune responses to ABVs remains to be shown.

BDV isolates from different mammal species exhibit a high level of nucleotide sequence conservation (*23*). Contrary to this finding, the nucleotide sequences of the so far detected ABVs show striking diversity. Kistler et al. (*8*) identified 5 distinct genetic groups of ABV sequences obtained from psittacinae birds from the United States and from Israel. The genetic characterization of 28 ABVs from Europe and 1 ABV from Australia showed that most of the viruses belong to the second and the fourth genetic group of the above-mentioned classification. Two viruses, however, formed a separate genetic group, which showed a similar genetic distance to the other 5 ABV groups as the distance to the representatives of BDV. Therefore, we believe that the establishment of a sixth group, termed ABV-6, in the preliminary classification of ABVs, is justified.

Another noteworthy observation is that ABV sequences obtained from certain tissue samples showed single or multiple nucleotide polymorphisms. The consistent results of repeated RT-PCRs and sequence determinations indicate that the polymorphism is not related to the in vitro amplifications and sequence determinations, but that the original samples contain mixtures of ABV sequences. One plausible explanation is that those birds were simultaneously infected with at least 2 ABV genotypes. Another possibility is that ABV exhibits quasispecies character; however, this situation would be rather unusual when compared to the characteristics of BDV. The mammalian bornaviruses represented by BDV form a separate branch within the bornaviruses and reflect strong sequence conservation as well as geographic clustering independently from year of isolation and species (*23*,*24*). The strain No/98 is an exception within this group because it originates from a horse outside of disease-endemic regions and possesses a higher sequence variation than the other mammalian bornaviruses, thereby being the closest relative to the ABVs thus far (*12*).

To this point, ABVs have been detected in at least 27 psittacine species from 4 different continents. Phylogenetic analyses do not indicate species-specificity or geographic clustering of the different ABV genogroups. The 2 ABV-6 genogroup sequences, which were detected in birds from Austria and Switzerland, are from geographically close areas; however, further investigations may show more widespread distribution of viruses from this genogroup as well. The wide geographic spread of different virus genotypes is most likely strongly influenced by the worldwide trade of the host species.

The diagnostic methods described in this article were found to be useful tools for the direct demonstration of viral RNA and antigens of different ABV genotypes in archived paraffin wax–embedded tissue samples as well as in fresh-frozen tissue samples. Because of the observed nucleotide sequence diversity, molecular techniques also have certain limitations. Of the 29 investigated birds, 2 gave positive IHC results with the polyclonal BDV serum, but gave negative results in both RT-PCR assays. These samples might contain ABVs that are genetically different from those of genogroups 2, 4, and 6.

The results of this study, as do those of another study published during the review of this manuscript (*25*), show that birds from Europe with PDD are consistently infected with ABVs and that they display viral antigen in neural and extraneural tissues. Issues that remain to be resolved, however, are whether psittacine birds are also the natural reservoir of these viruses or whether other species, in which disease probably does not develop upon infection, will be identified in the future. However, virus has not yet been detected in clinically healthy birds. The task of identifying natural reservoir hosts can be painstaking. For BDV, the search has been unsuccessful for decades, until Hilbe et al. suggested that a shrew species (*Crocidura leucodon*) may be a candidate for a natural reservoir host (*26*).

Acknowledgments

We are grateful to Peter Sandmeier, who submitted PDD-affected birds for further investigations; and to W. Ian Lipkin, Lothar Stitz, and Sybille Herzog for the generous gifts of BDV-specific antibodies. We also thank Helga Lussy, Christine Glatzer, and Karin Fragner for excellent technical assistance; and Klaus Bittermann for his help with the digital artwork.

This study was partially supported by the grant OTKA K67900. T B. is grantee of the "Bolyai János" postdoctoral fellowship grant of the Hungarian Academy of Science.

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References

1. Gregory CR, Latimer KS, Niagro FD, Ritchie BW, Campagnoli RP, Norton TM, et al. A review of proventricular dilatation syndrome. J Assoc Avian Vet. 1994;8:69–75.
2. Mannl A, Gerlach H, Leipold R. Neuropathic gastric dilatation in psittaciformes. Avian Dis. 1987;31:214–21. DOI: 10.2307/1590799
3. Daoust PY, Julian RJ, Yason CV, Artsob H. Proventricular impaction associated with nonsuppurative encephalomyelitis and ganglioneuritis in two Canada geese. J Wildl Dis. 1991;27:513–7.
4. Perpiñán D. Fernández-Bellon H, López C, Ramis A. Lymphoplasmacytic myenteric, subepicardial, and pulmonary ganglioneuritis in four nonpsittacine birds. J Avian Med Surg. 2007;21:210–4. DOI: 10.1647/1082-6742(2007)21[210:LMSAPG]2.0.CO;2
5. Berhane Y, Smith DA, Newman S, Taylor M, Nagy E, Binnington B, et al. Peripheral neuritis in psittacine birds with proventricular dilatation disease. Avian Pathol. 2001;30:563–70. DOI: 10.1080/03079450120078770
6. Vice CA. Myocarditis as a component of psittacine proventricular dilatation syndrome in a Patagonian conure. Avian Dis. 1992;36:1117–9. DOI: 10.2307/1591587
7. Busche R, Frese K, Weingarten M. Zur Pathologie des Macaw Wasting Syndroms. Verh-Ber Erkr Zoot. 1985;27:325–9.
8. Kistler AL, Gancz A, Clubb S, Skewes-Cox P, Fischer K, Sorber K, et al. Recovery of divergent avian bornaviruses from cases of proventricular dilatation disease: identification of a candidate etiologic agent. Virol J. 2008;5:88. DOI: 10.1186/1743-422X-5-88
9. Honkavuori KS, Shivaprasad HL, Williams BL, Quan PL, Hornig M, Street C, et al. Novel borna virus in psittacine birds with proventricular dilatation disease. Emerg Infect Dis. 2008;14:1883–6. DOI: 10.3201/eid1412.080984
10. Doneley RJ, Miller RI, Fanning TE. Proventricular dilatation disease: an emerging exotic disease of parrots in Australia. Aust Vet J. 2007;85:119–23. DOI: 10.1111/j.1751-0813.2007.00109.x
11. Pleschka S, Staeheli P, Kolodziejek J, Richt JA, Nowotny N, Schwemmle M. Conservation of coding potential and terminal sequences in four different isolates of Borna disease virus. J Gen Virol. 2001;82:2681–90.
12. Nowotny N, Kolodziejek J, Jehle CO, Suchy A, Staeheli P, Schwemmle M. Isolation and characterization of a new subtype of Borna disease virus. J Virol. 2000;74:5655–8. DOI: 10.1128/JVI.74.12.5655-5658.2000
13. Grabensteiner E, Ritter W, Carter MJ, Davison S, Pechhacker H, Kolodziejek J, et al. Sacbrood virus of the honeybee (*Apis mellifera*): rapid identification and phylogenetic analysis using reverse transcription-PCR. Clin Diagn Lab Immunol. 2001;8:93–104.
14. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.
15. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25:4876–82. DOI: 10.1093/nar/25.24.4876
16. Sasaki S, Ludwig H. In Borna disease virus infected rabbit neurons 100 nm particle structures accumulate at areas of Joest-Degen inclusion bodies. Zentralbl Veterinarmed B. 1993;40:291–7.
17. Malik TH, Kishi M, Lai PK. Characterization of the P protein-binding domain on the 10-kilodalton protein of Borna disease virus. J Virol. 2000;74:3413–7. DOI: 10.1128/JVI.74.7.3413-3417.2000
18. Grimm F. Der klinische Fall Neuropathische Magendilatation eines Ara. Tierarztl Prax. 1991;19:111–2.
19. Stitz L, Noske K, Planz O, Furrer E, Lipkin WI, Bilzer T. A functional role for neutralizing antibodies in Borna disease: Influence on virus tropism outside the central nervous system. J Virol. 1998;72:8884–92.
20. Pfannkuche H, Konrath A, Buchholz I, Richt JA, Seeger J, Müller H, et al. Infection of the enteric nervous system by Borna disease virus (BDV) upregulates expression of calbindin D-28k. Vet Microbiol. 2008;127:275–85. DOI: 10.1016/j.vetmic.2007.09.005
21. Herzog S, Kompter C, Frese K, Rott R. Replication of Borna disease virus in rats: age-dependent differences in tissue distribution. Med Microbiol Immunol (Berl). 1984;173:171–7. DOI: 10.1007/BF02122108
22. Stitz L, Schilken D, Frese K. Atypical dissemination of the highly neurotropic Borna disease virus during persistent infection in cyclosporine A–treated, immunosuppressed rats. J Virol. 1991;65:457–60.
23. Kolodziejek J, Dürrwald R, Herzog S, Ehrensperger F, Lussy H, Nowotny N. Genetic clustering of Borna disease virus natural animal isolates, laboratory and vaccine strains strongly reflects their regional geographical origin. J Gen Virol. 2005;86:385–98. DOI: 10.1099/vir.0.80587-0
24. Dürrwald R, Kolodziejek J, Muluneh A, Herzog S, Nowotny N. Epidemiological pattern of classical Borna disease and regional genetic clustering of Borna disease viruses point towards the existence of to-date unknown endemic reservoir host populations. Microbes Infect. 2006;8:917–29. DOI: 10.1016/j.micinf.2005.08.013
25. Rinder M, Ackermann A, Kempf H, Kaspers B, Korbel R, Staeheli P. Broad tissue and cell tropism of avian bornavirus in parrots with proventricular dilatation disease. J Virol. 2009;83:5401–7.
26. Hilbe M, Herrsche R, Kolodziejek J, Nowotny N, Zlinszky K, Ehrensperger F. Shrews as reservoir hosts of Borna disease virus. Emerg Infect Dis. 2006;12:675–7.

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Program to Eradicate Malaria in Sardinia, 1946–1950

Eugenia Tognotti

During 1946–1950, the Rockefeller Foundation conducted a large-scale experiment in Sardinia to test the feasibility of indigenous vector species eradication. The interruption of malaria transmission did not require vector eradication, but with a goal of developing a new strategy to fight malaria, the choice was made to wage a rapid attack with a powerful new chemical. Costing millions of dollars, 267 metric tons of DDT were spread over the island. Although malaria was eliminated, the main objective, complete eradication of the vector, was not achieved. Despite its being considered almost eradicated in the mid-1940s, malaria 60 years later is still a major public health problem throughout the world, and its eradication is back on the global health agenda.

In 1944, Sardinia was used as a test site for eradicating native malaria-carrying mosquitoes (*1*). During that year, the insecticide DDT (dichloro-diphenyl-trichloroethane) was sprayed inside houses to annihilate mosquitoes in Castel Volturno (*2*). During that spring, another trial was conducted in the Tiber Delta and Pontine marshes, where breeding sites of *Anopheles labranchiae,* the most common, abundant, and widely distributed vector in the Mediterranean basin, had increased dramatically after German troops strategically flooded a large area to hinder the movement of the Allied Armed Forces (*3*). In the face of a potential malaria outbreak, the Allied Malaria Control Commission studied the effect of the DDT spray, in the absence of other control measures, on anopheline density.

The operations in central Italy were under the direction of Paul F. Russell and Fred Soper, officers of the Rockefeller Foundation. Russell was a veteran of malaria-control campaigns and a graduate of the Harvard School of Public Health Soper was a public health administrator and epidemiologist who during 1939 and 1941 (*4*) had directed successful eradication campaigns of an invading vector, *A. gambiae,* in Brazil and Egypt. Attempting to eradicate the indigenous well-adapted mosquito species *A. labranchiae* was more difficult than attempting to eradicate an invading vector. Both believed that the miraculous effectiveness of DDT (*5*) opened up a dazzling new era for the study of malaria: DDT was highly effective against the parasite-carrying mosquitoes and interrupted the transmission of the malaria parasite. In addition DDT was inexpensive, considered safe, and easy to use.

In this climate of optimism, the Italian malariologist Alberto Missiroli convinced civil authorities in Italy to conduct a massive malaria control program. The United Nations Relief and Rehabilitation Administration (UNRRA) provided funds (*6*). The idea of large-scale eradication work in Sardinia took shape in a series of meetings involving Missiroli, the director of UNRRA for Italy, and Soper, who was a staunch advocate of the vector-eradication approach to malaria control.

In a July 1945 letter from Italy (*7*), Soper informed George H. Strode, scientific director of the International Health Division (IHD), that Missiroli was "very insistent that the first work" begin in Sardinia. He also reported on meetings with Colonel Reekie of the UNRRA. They had undertaken a rapid reconnaissance flight over Sardinia, and in conclusion Soper stated:

> …from available information and what little I had seen it appeared that anopheles eradication in Sardinia might be entirely feasible if the materials, transportation, money, and authority could be made available.

Last-minute decisions left little time for planning. In their haste, the Rockefeller Foundation staff underestimated the difficulties of the project. In addition, the rush to conclude the agreement with government representatives

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DOI: 10.3201/eid1509.081317

in Italy and with the High Commissioner for Sardinia led to a lack of clarity about the goal of the campaign (*8*). The aims of the IHD were entirely scientific, as was clearly explained in a letter from Strode to the UNRRA director in 1946: "The only reason that I was interested in the proposal was the fact that we were to attempt an eradication program among the indigenous species of anophelene" (*9*).

However, the public health authorities in Italy were interested in implementing a full-scale public health program and were willing to invest heavily in this endeavor and use their recovery funds. They were unlikely to have devoted so much interest to support a purely scientific experiment.

This ambiguity dragged on for 2 years. Ultimately, the project became a public health campaign against malaria. A change in the goal enabled the Regional Agency for the Anti-Anopheles Struggle in Sardinia (ERLAAS) team to convince the increasingly reluctant High Commissioners for Hygiene and Health to divert funds from the scant health budget toward the campaign. The story of the "Sardinian Project" (see online Technical Appendix, note 1, available from www.cdc.gov/EID/content/15/9/1460-Techapp.pdf), the greatest antimalaria effort in Europe since the discovery of the cycle of transmission of the disease, needs to be reexamined in the light of the recent debate about the new global malaria eradication strategy (*10*). This article, based on firsthand sources such as letters, memoranda, and diaries (*8*), concentrates on the objectives, errors, results, and final implications of the campaign.

Sardinia, a Malaria-Endemic Island

Malaria is believed to have been introduced to Sardinia by infected workers imported from North Africa after the Carthaginian conquest of Sardinia in 502 BC. The disease became endemic to this region during the medieval period (*11*), but since the classical ages, Sardinia had been tarred with the reputation as an "unhealthy island" (*12*) (online Technical Appendix, note 2). In the last decade of the nineteenth century, the average number of deaths caused by malaria on this island oscillated between 2,000 and 2,200 per year (in 1901, the island had a population of 795,793) (*13*). Sardinia kept the unfortunate primacy of being the most malaria-ridden region in Italy (Table 1) because of the high prevalence of *Plasmodium falciparum* and its associated high mortality rates. Rates were particularly high for children <5 years of age in highly malaria-endemic areas.

Economic and demographic development (*14*) was dramatically inhibited. Malaria infested the plains, which constituted the most fertile and least populated areas. The productivity of those affected with chronic disease was low, and they were unable to work during fever attacks (*15*). A decline in the mortality rate began after advanced antimalarial legislation (1900–1907) provided free quinine, which attacks malaria parasites in the bloodstream. In the 1920s and 1930s, the fascist regime carried out an indirect battle for eradication through its great land reclamation project, which used modern technology on a large scale for drainage and sanitation (*16*). The centralized "Italian way" produced a decline in malaria mortality rates, but rates also declined as a result of greater access to medical services by the rural population, the main reservoir of malaria in the past. Over 40 years, mortality rates declined from an average of 2,000 during 1890–1900 to 138 in 1939 and 88 in 1940. The decline in illness and death from malaria was interrupted only by the 2 world wars: in 1946, 74,600 malaria cases and 169 deaths were reported (*17*).

Table 1. Deaths from malaria, Italy and Sardinia

	No. deaths/100,000 inhabitants	
Years	Italy	Sardinia
1887–1889	58	300
1900–1902	59	298
1912–1914	6	43

At that time, malaria was still endemic to Sardinia. In 1947, an ERLAAS survey showed an overall spleen index (a measure of splenomegaly) of ≈21%; in many low-lying places, the index approached 100% (*18*). The effect of malaria on public health and economic growth was still severe; according to contemporary analyses, the vicious circle of poverty and disease could be broken only by eliminating malaria. Sardinia, therefore, appeared to be the ideal site. It was an island. In addition, the weakness of local power represented an additional advantage for a project that verged on being a military occupation of the territory.

However, there were enormous organizational and logistical problems. One was the sheer size of the island: 9,294 square miles, with mountainous massifs and ravines. Another was the fast-flowing streams that carried water into low-lying areas in the springtime, forming stagnant pools (*19*). The island was virtually devoid of internal communication systems, and the inhabitants lived almost exclusively in villages. Few local people had technical expertise, and it was not easy to recruit and train people as disinfectors, larva scouts, and sprayers or to find suitable staff to perform supply, transport, and administrative services. However, these obstacles did not hinder the IHD decision to implement the program. They feared that the ongoing crisis in UNRRA and the unstable political balance in Italy might ultimately impede their efforts.

On October 2, 1945, the Rockefeller Foundation formally agreed to collaborate in the project. A few weeks later, UNRRA allocated an initial sum of US $400,000 and approved the plan, in agreement with the Italian government and the Rockefeller Foundation. In April 1946, the IHD founded the special agency ERLAAS to implement the program. The first director was John Austin Kerr, and the medical entomologist was Thomas Aitken. The island was divided into divisions, sections, and sectors of 2.8

square miles, the basic geographic unit for antilarval spraying. The entomologic service headquarters were set up in Cagliari, and the chief executive officers operated from there. Workers on the ground were responsible for day-to-day operations in their specific localities and were crucial to the entire operation. The organization followed military principles of hierarchy and discipline. Scouts for larvae and pupae were given rewards for good work and penalized for sloppy performance.

Difficulties of the Antimalaria Campaign

Problems emerged even before the Sardinian Project began. Aitken's entomologic survey indicated that 3 principal species of *Anopheles* mosquitoes were in Sardinia: *A. labranchiae, A. algeriensis,* and *A. claviger*. Unlike tropical malaria-carrying mosquitoes that thrive close to villages, *A. labranchiae* "breeded usually in open water, but is often found in marshes and mountain streams" (*20*). According to some estimates, the number of water sites was somewhere between 1,000,000 and 1,200,000.

During their investigation, the entomologists faced the alarming fact that making a sharp distinction between the breeding places of *A. labranchiae* mosquitoes and those of other species was impossible. As a result, larva control was extremely difficult. The topography and the altitude of the various breeding sites meant that operations took longer and were more costly than forecast. Mules and donkeys (Figure 1) rather than jeeps had to be used to transport equipment. By mid-1946, it was already clear that eradication of the indigenous vector would be far more difficult than eradication of invaders such as *A. gambiae* mosquitoes in Brazil. Complaints in this regard made by superintendent Kerr to IHD headquarters were not well received. As well, their eagerness to achieve their objective encouraged them to overlook alarming information about the potential toxicity of DDT (online Technical Appendix, note 3), of which Fred Soper was aware as he insinuated in a letter, suggesting that there were "contraindications to the use of DDT as a larvicide as planned." At a meeting of agricultural entomologists in Riverside, California, USA, he had heard alarming news of rather high concentrations of DDT being found in animal milk. These were not "carefully studied observations," but he advised, "Caution may be indicated" (*21*).

In the summer of 1946, Kerr began warning about the need to study potential reinfestation after the campaign, stating that "a period of at least one full year" was needed for extensive ecological field studies (*22*). About Kerr's insistence, Soper wrote ironically to Strode that, "It is indeed to be regretted that the word 'ecology' was ever invented, or having been invented came to [Kerr's] attention" (*23*). In October 1946, the second ERLAAS Advisory Committee approved a new plan that included indoor residual spraying, trial larviciding, and all-out larviciding of the entire island.

Despite the optimism of IHD leaders, Kerr's misgivings increased. According to an account by the parasitologist O.R. McCoy, who visited Sardinia for the IHD, substantial problems had arisen as a result of Kerr's conviction that eradication was impossible. Kerr's concerns, and the tremendous difficulties of the eradication program, threatened to delay the operations by a whole year, at the risk of losing UNRRA funding. Hostility toward the organization increased. The aid to Eastern European countries was seen as a dangerous instrument that was facilitating the consolidation of communist governments. McCoy wrote, "Since additional funds depend upon UNRRA's recommendation it is essential that the budget for another year of work be approved." And, "The stake is too great," McCoy emphasized, "It was made very clear that the next few months during which UNRRA is still functioning are critical as far as ERLAAS is concerned."

Budget problems were becoming increasingly challenging, partly because of fluctuations in the value of the lira. Field experience had shown that the campaign would have to take longer than expected. Again, the Italian government reluctantly provided additional funds that permitted the program to continue. By June 1947, ≈85% of all villages and towns in Sardinia had been completely sprayed with DDT. The operations consisted of a single spraying of every room in every house, all outhouses, and isolated buildings in the countryside, including the ancient *nuraghi* (stone dwellings centered on a main tower or fortress) (*24*).

Effects of Early Cold War Tensions

Additional problems were created by the tensions of the Cold War (continuing state of conflict, tension, and competition after World War II) (*25*). On July 2, 1947, the

Figure 1. Donkeys used to transport equipment and larvicide in hilly territory, Sardinia, 1948–1950. Photograph by Wolfgang Suschitzky. Reprinted with permission from Istituto Etnografico della Sardegna.

Sardinian edition of l'Unità, a newspaper that served as the mouthpiece of the Communist Party, wrote that ERLAAS was creating a neo-fascist organization in Sardinia, with a hierarchical, almost military, structure that had 600 vehicles and cells (organized groups) in the villages. This information appeared in the International Herald Tribune on July 21, 1947. In the following months, communists began promoting disruption of the execution of the Marshall Plan by means of open confrontation with local governments. This situation prompted the IHD to transform the original objective and proceed more swiftly. "The eyes of the world were upon the Anopheles eradication," and it was of prime importance to move forward at all costs before a crisis ensued (*26*). However, Kerr's conviction that the eradication of *A. labranchiae* mosquitoes was not feasible (*27*) was problematic. Finally, in a dramatic letter to Strode, the superintendent commented "I do not have either the mental or physical stamina for this task, which I am convinced is certain to fail." The frantic correspondence among the chief executives indicated that they feared that the campaign was destined for failure, while they were intending to present positive results at the first meeting of the World Health Organization Expert Committee on Insecticides, which would take place in Cagliari in May 1948. In September 1947, the following dramatic scenario played out, including a letter from Bauer to Strode:

> It would be a tragedy if the project was abandoned now without a thorough trial. It would open us to all sort of criticism, especially in view of the fact that a large sum of money which did not belong to us in the first place has already been spent; Italian communists would jump on this occasion (*28*).

The ultimate decision was that Kerr should be replaced. Under the new superintendent, John Logan, the operations continued with the planned residual spraying against adult mosquitoes. A quarantine service was set up, and ships and planes arriving in Sardinia were inspected (*29*).

Political tensions grew as the elections of April 1948 approached. The US government intervened in Italy to prevent the Communists and the Socialists from winning election funding. The Truman administration declared that no further help from the European Recovery Program (Marshall Plan) would be given to the country if the Communist party won the elections (online Technical Appendix, note 4).

Communist press attacks on the Rockefeller Foundation increased. Some newspapers wrote that the ERLAAS vehicles were secretly armed and equipped to "take over" Sardinia. A radio report from northern Italy claimed that ERLAAS was paving the way for the transformation of the island into an enormous US air base (*30*). Furthermore, antagonism to the larviciding was growing, and legal actions for damages were pending.

Overall, ERLAAS operations were welcomed by the people of Sardinia. The inhabitants of the rural areas appreciated the abatement of mosquitoes and houseflies. Exhortations to the disinfectors appeared in verse on rocks and house walls. The few criticisms of the campaign concerned the "violence of the method."

In 1948, a sociologist a report on communism in Sardinia concluded that "the popular Front deputies at Rome could cause some outcry over the allocation of government controlled funds for equipment" (*31*). At this time, the staff of the Sardinian Project did not speak of "an eradication program among indigenous species of anophelines" but of "a large project which is one of the most important public health in the world today" (*32*). Various leaflets were used

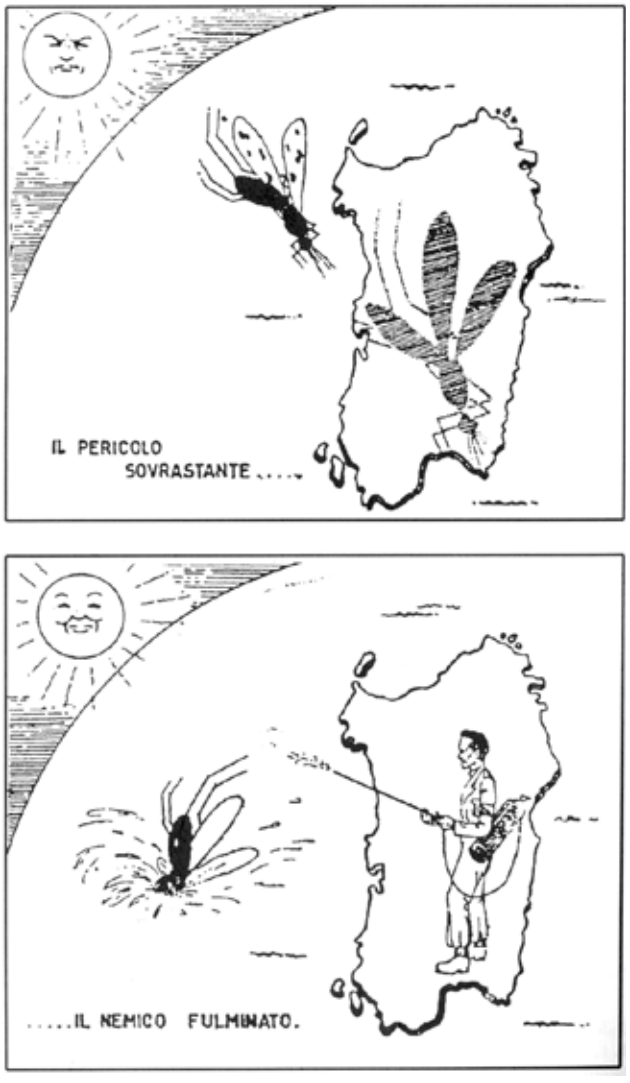

Figure 2. Poster by the Regional Agency for the Anti-Anopheles Struggle in Sardinia. Photograph by Wolfgang Suschitzky. Reprinted with permission from Istituto Etnografico della Sardegna.

to demonstrate the beneficial effects of the campaign. One showed "before" and "after" images of Sardinia; "before" pictured a frowning sun and a giant mosquito, and "after" featured a smiling sun and an island free of mosquitoes, wiped out by a jet of DDT (Figure 2).

At the end of 1948, the campaign entered its final phase. In the summer of 1948, the last offensive against *Anopheles* larvae (Figure 3) was launched as sort of a "Normandy Landing" with an army of 30,000 men. Foci were cleaned with long-handled billhooks, vegetation was cut back, 100,000 acres of swampland were drained, and tons of insecticide were spread over the island by aircraft and helicopters (Figure 4). At the height of the campaign, the weekly amount of pure DDT spread was about 3,250 kg. Approximately 110 km^2 of water had been treated with a dose of 30 mg/m^2 (*33*). At the end of that year, the management of ERLAAS announced that the number of breeding places of *Labranchiae* mosquitoes had been drastically reduced. The presumed reduction was 99.93%. The remaining positive foci were mainly in isolated areas (*34*). In 1950, for the first time in the history of Sardinia, no new cases of the disease were reported on the island (Table 2).

The first large-scale attempt to rid a malaria-endemic area of indigenous mosquitoes had not succeeded, but it did free Sardinia from malaria. Emphasizing this outcome enabled the hierarchy of the IHD to maintain the concept of eradication, which prevailed in 1955 in the Eighth World Health Assembly, and they voted to adopt DDT as a primary tool in the fight against malaria (*35*).

IHD leaders slowly created a story of success (*36*). Writing to Missiroli, Paul Russell exalted the fact that "the local health authorities could forever keep it under control, while the first large areas previously infested with the disease could be reclaimed and cultivated." He also emphasized the scientific results:

> If the ERLAAS proves that it is not feasible to attempt complete eradication of a tenacious indigenous species like *A. labranchiae* as a measure of malaria control, such an answer will be of great value to the scientific world, because on all sides we hear the cry "eradicate the mosquito" (*37*).

Conclusions

During the campaign, under the pressure of various factors, the initial ambitious purpose had changed: Sardinia, at the end, was free from the disease, not from the vectors that remained. However, vector breeding places were drastically reduced by 99.93%.

The widespread use of DDT was not required, considering the potential negative effect on the environment and on persons. To interrupt malaria transmission, indoor DDT spraying, as already demonstrated in peninsular areas where the chemical was sprayed in small amounts on the house walls, would have been sufficient.

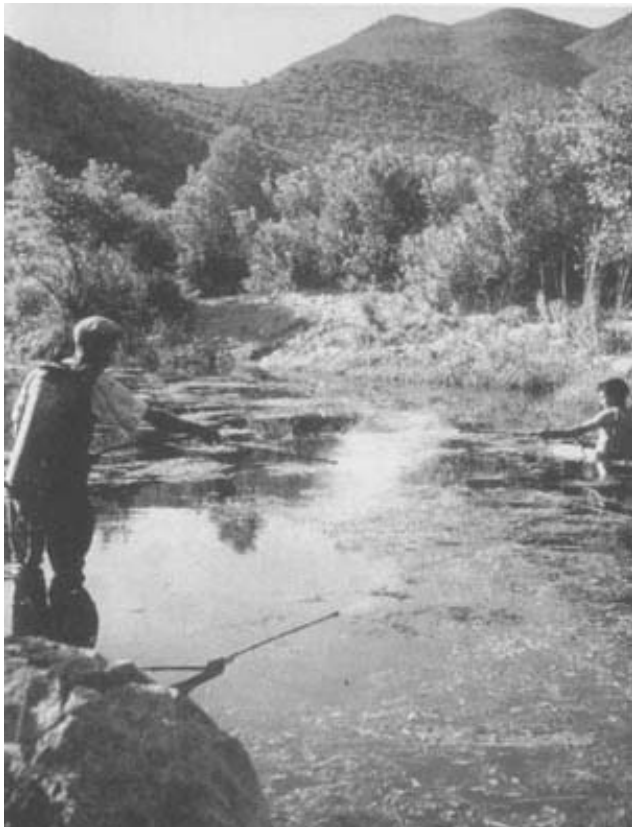

Figure 3. Larviciding. Photograph by Wolfgang Suschitzky. Reprinted with permission from Istituto Etnografico della Sardegna.

At the 60th anniversary of the end of the campaign, a risk-to-benefit assessment was possible. It is an established fact that the eradication of malaria contributed powerfully to the subsequent socioeconomic development and public health of the island.

With respect to the possible long-term effects of DDT, a team of Sardinian researchers recently conducted studies to determine whether DDT has negatively affected the health of the human population of the island. On the basis of statistics on births and stillbirths in the prewar and postwar years (1945–1954), widespread use of DDT apparently did not affect stillbirth rates, infant mortality rates, or the male:female ratio of newborns (*38*). With regard to the potential carcinogenicity of DDT, the results of the most recent follow-up study of deaths among 4,552 male workers exposed to DDT demonstrated little evidence of a link between occupational DDT exposure and death from any of the cancers previously associated with exposure to this chemical (e.g., pancreatic cancer) (*39*). The researchers of

Figure 4. Aerial spraying of DDT in Sardinia, 1948. Photograph by Wolfgang Suschitzky. Reprinted with permission from Istituto Etnografico della Sardegna.

this study argued that expansion of the cohort and collection of information are needed to clarify these findings. No studies of the environmental effects have been conducted.

The lessons learned from the Rockefeller Foundation antimalarial campaign in Sardinia have contemporary relevance in discussions of DDT-based malaria control strategies around the world. Nevertheless, although DDT played an important role in the liberation of the island from malaria, it was not sufficient alone to accomplish the task. The benefits of this enormous expenditure of funds were cast-iron (inflexible) organization, exceptional technical and scientific expertise, and continuity in mosquito control efforts maintained by the regional government for decades after conclusion of the campaign. Geographic isolation also played a role. Furthermore, the support of UNRRA and of the Italian High Commissioner for Health, as well as the ability and experience of the Rockefeller Foundation staff, neutralized the considerable obstacles of lack of technical resources, expertise, and infrastructure on the ground. An additional factor was the favorable attitude of the local community, which had grown accustomed for decades to fighting malaria with quinine and with land reclamation projects that reduced the mosquito habitat.

Table 2. Cases of malaria, by year, Sardinia, 1946–1952

Year	No. cases (relapses)
1946	74,641
1947	39,303
1948	15,121
1949	1,314
1950	0 (44)
1951	9 (8)
1952	0

In conclusion, the Rockefeller Foundation antimalarial campaign in Sardinia was an important step in the development of malaria control policies in the 20th century. It displays the various approaches to the control of malaria and contributes important lessons for the ongoing debate over possible solutions to the terrible problem of malaria and the difficult challenge of eliminating it from the modern world (*40*).

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References

1. Stapleton DH. Lessons of history? The anti-malaria strategies of the International Health Board and the Rockefeller Foundation from the 1920s to the era of DDT. Public Health Rep. 2004;119:206–15.
2. Soper Fred L, Knipe FW, Casini G, Riehl Louis A, Rubino A. Reduction of *Anopheles* density effected by the preseason spraying of building interiors with DDT in kerosene, at Castel Volturno, Italy, in 1944–1945 and in the Tiber Delta in 1945. Am J Trop Med Hyg. 1947;27:177–200.
3. Snowden F. The conquest of malaria: Italy, 1900–1962. New Haven (CT): Yale University Press; 2006.
4. Packard RM, Gadelha P. A land filled with mosquitoes: Fred Soper, the Rockefeller Foundation and the *Anopheles gambiae* invasion of Brazil, 1932–1939. Parassitologia. 1994;36:197–214.
5. Missiroli A. *Anopheles* control in the Mediterranean area. In: Proceedings of the 4th International Congress on Tropical Medicine and Malaria; 1948 May 10–18; Washington, DC; 1566–75.
6. Gladwell M. Fred Soper and the Global Malaria Eradication Programme. J Public Health Policy. 2002;23:479–97. DOI: 10.2307/3343244
7. Rockefeller Foundation Archives. Letter. 1945 Jul 28; series 700, folder 103, record group 1.2.
8. Rockefeller Foundation Archives. Committee meeting minutes; Cagliari (Sardinia, Italy) 1946 May 14; series 700, folder 104, box 12, record group 1.2.
9. Rockefeller Foundation Archives. Letter from GH Strode to WA Sawyer. 1946 Oct 10; series 700, folder 105, box 12, record group 105.
10. Feachem R, Sabot O. A new global malaria eradication strategy? Lancet. 2008;371:1633–5. DOI: 10.1016/S0140-6736(08)60424-9
11. Sallares R, Bouwman A, Anderung C. The spread of malaria to southern Europe in antiquity: new approaches to old problems. Med Hist. 2004;48:311–28.

12. Brown PJ. Malaria in Nuragic, Punic, and Roman Sardinia: some hypotheses. In: Balmouth MS, Rowland RJ editors. Studies in Sardinian archeology. Ann Arbor (MI): University of Michigan Press; 1984. p. 209–35.
13. Tognotti E. Per una storia della malaria in Italia. Il caso della Sardegna, 2nd ed. Milano (Italy): Franco Angeli; 2008. p. 222.
14. Brown PJ. Malaria, miseria, and underpopulation in Sardinia: the malaria blocks development cultural model. Med Anthropol. 1997;17:239–54.
15. Alivia M, Lustig A, Sclavo A. Campagna antimalarica in Sardegna nel 1911. Firenze (Italy): Soc. Tipografica Fiorentina; 1912.
16. Schmidt CT. Land reclamation in fascist Italy. Polit Sci Q. 1937;52:340–63.
17. Cause di morte 1887–1985, Istituto Centrale di Statistica, Roma: azienda beneventana tipografica editoriale; 1953; p. 193.
18. Hackett LW. Spleen measurement in malaria. Journal of the National Malaria Society. 1944;3:121–33.
19. Ente regionale per la lotta anti-anofelica in Sardegna Archives. Bollettino dell'Ente Regionale per la Lotta Antianofelica in Sardegna. 1948 Sep 1; Cagliari, Sardinia (Italy).
20. Rockefeller Foundation Archives. Final commentary. The Sardinian Project; series 700, folder 138, box 16, record group 1.2.
21. Rockefeller Foundation Archives. Letter to Strode. 1946 Jul 8; series 700, folder 104, box 12, record group 1.2.
22. Rockefeller Foundation Archives. Letter from Kerr to Strode. 1946 Jun 6; folder 104, box 12, series 700, record group 1.2.
23. Rockefeller Foundation Archives. Letter from Soper to Strode. 1946 Jun 22; folder 104, box 12, series 700.
24. Logan J. The Sardinian Project: an experiment in the eradication of an indigenous malarious vector. Baltimore: Johns Hopkins Press; 1953.
25. Gaddis JL. The Cold War: a new history. New York: Penguin Books; 2005.
26. Rockefeller Foundation Archives. Report and enclosures. 1947 May 4; series 700, folder 22, box 13, record group 1.
27. Rockefeller Foundation Archives. Letter from Kerr to Strode. 1947 Sep 8; series 700, folder 111, box 13, record group 1.2.
28. Rockefeller Foundation Archives. Confidential letter from Bauer to Strode. 1947 Aug 28; series 700, folder 111, box 13, record group 1.2.
29. Garrett Jones C. Anopheles eradication in Sardinia. Rockefeller Foundation Archives; series 700, folder 113, box 13, record group 1.2.
30. Tognotti E. Americani, comunisti e zanzare. Il piano di eradicazione della malaria in Sardegna tra scienza e politica negli anni della guerra fredda (1946–50). Sassari (Italy): Edes; 1995.
31. Chesney MR. Report on Communism to Rockefeller Foundation. 1949 Jan 3; Rockefeller Foundation Archives; series 700, folder 113, box 13, record group 1.2.
32. Rockefeller Foundation Archives. Letter from Logan to the High Commissioner for Health and Hygiene. 1947 Oct 13; series 700, folder 113, box 13, record group 1.2.
33. Garrett Jones C. *Anopheles* eradication in Sardinia. Rockefeller Foundation Archive, p. 8.
34. Rockefeller Foundation Archives. Confidential memorandum to JBG from 13 PFR regarding ERLAAS; folder 114, box 13, series 700, record group 1.2.
35. Farley J. Mosquitoes or malaria? Rockefeller campaigns in the American South and Sardinia. Parassitologia. 1994;36:165–73.
36. Rockefeller Foundation Archive. Letter from PF Russel to Missiroli. Series 700, folder 21, box 14, record group 1.2.
37. Cocco P, Blair A, Congia P, Saba G, Ecca AR, Palmas C. Long-term health effects of the occupational exposure to DDT. A preliminary report. Ann N Y Acad Sci. 1997;837:246–56. DOI: 10.1111/j.1749-6632.1997.tb56878.x
38. Cocco P, Fadda D, Billai B, D'Atri M, Melis M, Blair A. Cancer mortality among men occupationally exposed to dichlorodiphenyltrichloroethane. Cancer Res. 2005;65:9588–94. DOI: 10.1158/0008-5472.CAN-05-1487
39. Packard RM. 'No other logical choice': global malaria eradication and the politics of international health in the post-war era. Parassitologia. 1998;40:217–29.
40. Feachem RG, Sabot OJ. Global malaria control in the 21st century: A historic but fleeting opportunity. JAMA. 2007;297:2281–4. DOI: 10.1001/jama.297.20.2281

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Genetic Differences between Avian and Human Isolates of *Candida dubliniensis*

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When *Candida dubliniensis* isolates obtained from seabird excrement and from humans in Ireland were compared by using multilocus sequence typing, 13 of 14 avian isolates were genetically distinct from human isolates. The remaining avian isolate was indistinguishable from a human isolate, suggesting that transmission may occur between humans and birds.

Candida dubliniensis is an opportunistic yeast species phenotypically and genetically closely related to *C. albicans*, the most common cause of *Candida* infection. However, *C. dubliniensis* is less pathogenic and is most commonly associated with superficial infection in immunocompromised persons. Although *C. albicans* has frequently been isolated from avian and animal sources (*1–4*), the recent study by Nunn et al. identified *C. dubliniensis* from a nonhuman source (*5*). These isolates were obtained from the surface of *Ixodes uriae* ticks that lived in cracks filled with seabird excrement at 2 locations at a seabird breeding colony on Great Saltee Island off the southeastern coast of Ireland.

Multilocus sequence typing (MLST) is an informative tool for investigating the population structure and epidemiology of many bacterial and fungal species (*6*). We have used MLST to show that *C. dubliniensis* has less genetic diversity than *C. albicans* and that *C. dubliniensis* isolates comprise 3 distinct clades (C1, C2, and C3), which correspond to described internally transcribed spacer (ITS) region genotypes 1–4 (*7*). Two other research groups recently used MLST to show genetic differences between *C. albicans* isolates from humans and animals (*3*,*4*). The purpose of our study was to use MLST, the presence or absence of a previously identified polymorphism in the *CDR1* gene (*8*), and mating type analysis to determine genetic relatedness between avian-associated and human *C. dubliniensis* isolates and whether avian-associated isolates are a source of human opportunistic infections.

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DOI: 10.3201/eid1509.081660

The Study

To obtain avian-associated *C. dubliniensis* isolates from a novel geographic site, fresh seabird excrement was sampled from the campus of Trinity College Dublin, ≈150 km north of Great Saltee Island by using nitrogen-gassed VI-PAK sterile swabs (Sarstedt-Drinagh, Wexford, Ireland). Samples were plated within 2 h of collection on CHROMagar *Candida* medium (CHROMagar, Paris, France), incubated at 30°C for 48 h, and identified as described (*7*,*9*–*12*). Three new *C. dubliniensis* isolates were obtained from 134 fecal samples. Like isolates from Great Saltee Island (*5*), these 3 isolates obtained directly from freshly deposited herring gull (*Larus argentatus*) excrement were ITS genotype 1 (*13*). Because the isolates originally described by Nunn et al. (*5*) were obtained from the surface of ticks living in avian excrement, avian-associated isolates refers to avian excrement–associated isolates. The avian-associated isolates were compared with 31 human *C. dubliniensis* strains belonging to MLST clade C1 as previously reported (*7*), and 5 additional *C. dubliniensis* clade C1 human isolates from Ireland (Table).

Isolates were assigned a diploid sequence type (DST) on the basis of genotype numbers for the 8 loci in the recommended *C. dubliniensis* MLST typing scheme (*7*) (Table). Six new DSTs were identified in 13 of 14 avian-associated isolates because of the identification of 2 new *exZWF1b* alleles that were found exclusively in avian- associated isolates. DST2 was the only previously identified DST (isolate AV7, Table). DST 31 was the most frequently (5/14 isolates) found DST in avian-associated *C. dubliniensis* isolates, all 5 of which were from Great Saltee Island (*5*). Four isolates belonged to DST 27, three from Great Saltee Island and 1 from Dublin (Table).

Polymorphic sites (n = 36) from the 8 MLST loci (*7*) of all 50 clade C1 human and avian-associated *C. dubliniensis* isolates were concatenated and used to construct a neighbor-joining tree (MEGA software program version 3.1 [*14*]), which included all known clade C1 DSTs identified. Thirteen of 14 avian-associated *C. dubliniensis* isolates, 11 from Great Saltee Island (*5*) and 2 from Dublin, formed a distinct subgroup within clade C1 (Figure, panel A). This same subgroup was also identified in trees generated by using the unweighted pair group method with arithmetic mean, maximum parsimony, and maximum likelihood, and based on related sequence types (BURST) analysis. To test

Table. Newly investigated avian-associated and human isolates of *Candida dubliniensis*, Ireland*

Isolate	Source	Year of isolation	Location	DST†	Mating type	TAG	Reference
SL411	*Ixodes uriae* ticks	2007	GSI	27	aa	+	(*5*)
SL422	*I. uriae*	2007	GSI	27	aa	+	(*5*)
SL370	*I. uriae*	2007	GSI	27	aa	+	(*5*)
SL410	*I. uriae*	2007	GSI	29	aa	+	(*5*)
SL375-I	*I. uriae*	2007	GSI	31	aa	+	(*5*)
SL375-II	*I. uriae*	2007	GSI	31	aa	+	(*5*)
SL397	*I. uriae*	2007	GSI	31	aa	+	(*5*)
SL414	*I. uriae*	2007	GSI	31	aa	+	(*5*)
SL495	*I. uriae*	2007	GSI	33	aa	+	(*5*)
SL509	*I. uriae*	2007	GSI	30	aa	+	(*5*)
SL522	*I. uriae*	2007	GSI	31	aa	+	(*5*)
AV5	*Larus argentatus*‡	2008	TCD	29	aa	+	This study
AV6	*L. argentatus*	2008	TCD	27	aa	+	This study
AV7	*L. argentatus*	2008	TCD	2	aα	+	This study
CD06032	Human, oral	2006	Ireland	36	αα	–	This study
CD06027	Human, oral	2006	Ireland	1	aα	+	This study
CD0512	Human, oral	2005	Ireland	37	aα	–	This study
CD524	Human, oral	1997	Ireland	35	aα	–	(*13*)
CD505	Human, oral	1989	Ireland	28	αα	+	(*13*)

*All 19 isolates were internal transcribed spacer genotype 1 and belonged to multilocus sequence typing clade C1. DST, diploid sequence type; TAG, TAG polymorphism; GSI, Great Saltee Island off the southeastern coast of Ireland; TCD, Trinity College Dublin.
†Assigned to each isolate according the recommended multilocus sequence typing scheme for *C. dubliniensis* (scheme D) (*7*). All DSTs, except for DST1 and DST 2, are new.
‡Herring gull.

for genetic separation between human and avian-associated isolates obtained from the same country, a neighbor-joining tree was constructed by using 13 avian-associated and human clade C1 isolates from Ireland, each of which represented unique DSTs. The tree showed the robustness of the avian-associated subgroup within a population of human isolates from the same country, and the distribution of avian-associated and human isolates differed significantly ($p = 0.025$, by Fisher exact test) (www.exactoid.com/fisher/index.php) (Figure, panel B).

The prevalence of a common point mutation, previously identified in the *CDR1* gene of some ITS genotype 1 isolates, was determined for avian and human *C. dubliniensis* isolates as described previously (*8*). All 14 avian-associated isolates had the TAG polymorphism (Table) compared with 19 (53%) of 36 human clade C1 isolates. The mating types of the isolates were determined by multiplex PCR amplification by using 2 pairs of mating type locus (MTL)–specific primers. A 535-bp amplimer was generated with primers MTLa1-F (5′-TGAAAATGAAGACAATGCGA-3′) and MTLa1-R (5′-CATCTTTTTCTGCTATCAATTC-3′) in the presence of MTL type a DNA, and a 615-bp product resulted from primers MTLα1-F (5′-ATGAATTCACATCTGGAGGC-3′) and MTLα1-R (5′-CTGTTAATAGCAAAGCAGCC-3′) in the presence of MTL type α DNA. Amplification reactions contained 10 pmol of each of the forward and reverse primers, 2.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, pH 9.0 at 25°C, 10 mmol/L KCl, 0.1% (vol/vol) Triton X-100, 1.25 U GoTaq polymerase (Promega, Madison, WI, USA), and 25 μL of template DNA in a total volume of 50 μL. Cycling conditions were at 94°C for 10 min; 30 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min; and a final step at 72°C for 10 min.

Of the 14 avian excrement–associated isolates, 13 were MTLa homozygous (a/a), (Table). Only 4 (11.1%) of 36 human clade C1 isolates were homozygous for MTLa; 28 (77.7%) of 36 were heterozygous for MTL (a/α). A previous study also reported that 17 (20.7%) of 82 human *C. dubliniensis* isolates were homozygous for MTLa (*15*). The gull isolate AV7 was indistinguishable from human isolates CD71, SA115, and CM1 by MLST and has the same mating type (a/α). We propose that AV7 may be a human isolate that colonized a gull scavenging on the Trinity College Dublin campus. The TAG polymorphism and mating type data from the avian-associated isolates suggest a highly clonal population.

Conclusions

The avian-associated *C. dubliniensis* isolates investigated belong to MLST clade C1, which includes most human isolates. However, most (13/14) of the avian-associated isolates form a distinct subgroup within this clade, which suggests that despite the low level of variation within *C. dubliniensis*, a distinct avian subpopulation may be present. This suggestion is supported by the observation that 2/3 isolates (AV5 and AV6) obtained in Dublin belonged to the same subpopulation (defined by MLST, *CDR1*, and MTL loci) as isolates obtained from Great Saltee Island, which is 150 km from Dublin. Similar data suggesting ge-

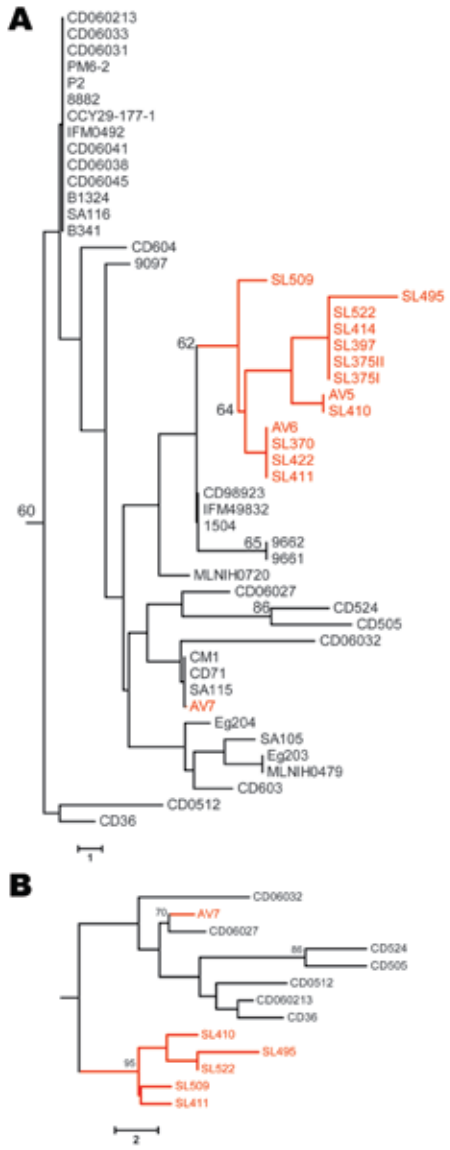

Figure. Neighbor-joining trees based on the polymorphic sites in *Candida dubliniensis* multilocus sequence typing (MLST) sequences. Bootstrap values >60% are indicated at cluster nodes. Avian-associated isolates are indicated in red. Numbers of polymorphic sites in isolates are indicated by scale bars. A) Isolates of MLST clade C1 defined by McManus et al. (*7*) showing location of avian-associated isolates in relation to human isolates in the same clade; human isolates were originally obtained in many countries. B) Neighbor-joining tree based on polymorphic sites in MLST sequences for each of 13 internal transcribed spacer genotype 1 *C. dubliniensis* isolates, 7 of which were obtained from humans in Ireland and 6 from seabird excrement in Ireland. Isolates that had identical diploid sequence types (DSTs) were not included in the tree so that only 1 of each DST is included. Tree displays the robustness of the avian-associated subgroup of isolates within a population of similar human-associated isolates from the same region. The rate of heterozygosity among human and avian-associated clade C1 isolates was 1.6 and 1 heterozygous site per DST, respectively, from 36 polymorphic sites, which indicated that avian-associated isolates were more clonal.

netic separation and differential clade distribution between human and animal populations of *C. albicans* have been reported (*3*,*4*). The presence of the avian-associated subgroup within the most predominant clade (C1), which had previously only been identified in human isolates, and the close genetic relatedness between isolates, in particular gull isolate AV7, suggests that transmission between the 2 hosts can occur. However, in this instance the most likely direction of transfer is from human to bird.

Acknowledgments

We thank Oscar Merne for assistance with collection of avian excrement samples and identification of specific species of birds at sampling sites.

This study was supported by the Microbiology Research Unit, Dublin Dental School and Hospital.

Ms McManus is a doctoral candidate in the Microbiology Research Unit at Dublin Dental School and Hospital, Trinity College Dublin. Her research interests focus on analysis of the population structure of the novel yeast pathogen *C. dubliniensis* by using MLST.

References

1. Buck JD. Isolation of *Candida albicans* and halophilic *Vibrio* spp. from aquatic birds in Connecticut and Florida. Appl Environ Microbiol. 1990;56:826–8.
2. Edelmann A, Kruger M, Schmid J. Genetic relationship between human and animal isolates of *Candida albicans.* J Clin Microbiol. 2005;43:6164–6. DOI: 10.1128/JCM.43.12.6164-6166.2005
3. Wrobel L, Whittington JK, Pujol C, Oh SH, Ruiz MO, Pfaller MA, et al. Molecular phylogenetic analysis of a geographically and temporally matched collection of *Candida albicans* isolates from humans and nonmigratory wildlife in central Illinois. Eukaryot Cell. 2008;7:1475–86. DOI: 10.1128/EC.00162-08
4. Jacobsen MD, Bougnoux ME, d'Enfert C, Odds FC. Multilocus sequence typing of *Candida albicans* isolates from animals. Res Microbiol. 2008;159:436–40. DOI: 10.1016/j.resmic.2008.05.003
5. Nunn MA, Schaefer SM, Petrou MA, Brown JR. Environmental source of *Candida dubliniensis.* Emerg Infect Dis. 2007;13:747–50.
6. Odds FC, Jacobsen MD. Multilocus sequence typing of pathogenic *Candida* species. Eukaryot Cell. 2008;7:1075–84. DOI: 10.1128/EC.00062-08
7. McManus BA, Coleman DC, Moran G, Pinjon E, Diogo D, Bougnoux ME, et al. Multilocus sequence typing reveals that the population structure of *Candida dubliniensis* is significantly less divergent than that of *Candida albicans.* J Clin Microbiol. 2008;46:652–64. DOI: 10.1128/JCM.01574-07
8. Moran G, Sullivan D, Morschhauser J, Coleman D. The *Candida dubliniensis CdCDR1* gene is not essential for fluconazole resistance. Antimicrob Agents Chemother. 2002;46:2829–41. DOI: 10.1128/AAC.46.9.2829-2841.2002
9. Coleman D, Sullivan D, Harrington B, Haynes K, Henman M, Shanley D, et al. Molecular and phenotypic analysis of *Candida dubliniensis*: a recently identified species linked with oral candidosis in HIV-infected and AIDS patients. Oral Dis. 1997;3(Suppl 1):S96–101.

10. Al Mosaid A, Sullivan DJ, Coleman DC. Differentiation of *Candida dubliniensis* from *Candida albicans* on Pal's agar. J Clin Microbiol. 2003;41:4787–9. DOI: 10.1128/JCM.41.10.4787-4789.2003
11. Pincus DH, Coleman DC, Pruitt WR, Padhye AA, Salkin IF, Geimer M, et al. Rapid identification of *Candida dubliniensis* with commercial yeast identification systems. J Clin Microbiol. 1999;37:3533–9.
12. Donnelly SM, Sullivan DJ, Shanley DB, Coleman DC. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. Microbiology. 1999;145:1871–82. DOI: 10.1099/13500872-145-8-1871
13. Gee SF, Joly S, Soll DR, Meis JF, Verweij PE, Polacheck I, et al. Identification of four distinct genotypes of *Candida dubliniensis* and detection of microevolution in vitro and in vivo. J Clin Microbiol. 2002;40:556–74. DOI: 10.1128/JCM.40.2.556-574.2002
14. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform. 2004;5:150–63. DOI: 10.1093/bib/5.2.150
15. Pujol C, Daniels KJ, Lockhart SR, Srikantha T, Radke JB, Geiger J, et al. The closely related species *Candida albicans* and *Candida dubliniensis* can mate. Eukaryot Cell. 2004;3:1015–27. DOI: 10.1128/EC.3.4.1015-1027.2004

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Rickettsia parkeri in *Amblyomma americanum* Ticks, Tennessee and Georgia, USA

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To determine the geographic distribution of the newly recognized human pathogen *Rickettsia parkeri,* we looked for this organism in ticks from Tennessee and Georgia, USA. Using PCR and sequence analysis, we identified *R. parkeri* in 2 *Amblyomma americanum* ticks. This rickettsiosis may be underdiagnosed in the eastern United States.

The most commonly reported rickettsial human pathogen in the United States is *Rickettsia rickettsii,* the causative agent of Rocky Mountain spotted fever (RMSF). First identified in 1937 (*1*), *R. parkeri* has been recognized as a human pathogen only since 2004, when it was isolated from an eschar on a serviceman from Virginia (*2*), although an Ohio patient suspected to have RMSF died of *R. parkeri* rickettsiosis in 1990 (*3*). Little is known about the geographic distribution of *R. parkeri* in the United States or the epidemiology of the disease it causes. The primary vector of *R. parkeri* is thought to be the Gulf Coast tick (*Amblyomma maculatum*), and naturally infected Gulf Coast ticks have been reported in numerous southeastern states (*4*). Experimentally, *A. americanum* ticks can maintain and transmit *R. parkeri* (*5*); thus, this tick species, which is more abundant than *A. maculatum* and bites humans aggressively (*6*), might contribute to *R. parkeri* transmission. To determine the geographic distribution of *R. parkeri*, we examined ticks collected in Tennessee and Georgia.

The Study

In Georgia, during 2005–2006, residents were encouraged to submit ticks to the state's Division of Public Health for identification and testing for various tick-borne pathogens. We studied only ticks that had been attached to persons. Ticks were individually homogenized with metal beads and resuspended in 225 μL of phosphate-buffered saline. DNA was extracted from 100 μL of the homogenate by using a QIAamp DNA Micro Kit (QIAGEN Inc, Valencia, CA, USA) according to the manufacturer's instructions. We conducted a nested PCR targeting the 17-kDa gene (*7*) and used bidirectional sequencing at the Integrated Biotechnology Laboratories (University of Georgia, Athens, GA, USA) to confirm positive results.

In Tennessee, from April 2007 through September 2008, the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, and the Tennessee Department of Health collected *A. americanum* and *A. maculatum* ticks from 31 counties by examining wild animals and dragging flannel sheets through vegetation. Ticks were stored in 100% ethanol and were sent to the Tennessee Department of Health Vector-Borne Diseases Laboratory for identification of species and life stage and detection of pathogens by molecular testing. A PCR targeting the outer membrane protein A (*rOmpA*) gene of spotted fever group (SGF) rickettsiae was conducted as previously described, using primers Rr190.602n and Rr190.70p (*8*). To identify the rickettsiae species, we subjected positive samples to a restriction fragment length polymorphism (RFLP) assay by digestion of the amplicons with *Rsa*I (Promega, Madison, WI, USA) and *Pst*I (Fermentas, Glen Burnie, MD, USA) enzymes at 37°C for 2 h. Digested fragments were subjected to electrophoresis on 10% polyacrylamide gels. To confirm species identification, we purified representative positive PCR products with Exosap-It (USB Corporation, Cleveland, OH, USA), sequenced at the Tennessee Department of Health Laboratory Services (Nashville, TN, USA) and entered into the National Center for Biotechnology Information BLAST database (www.blast.ncbi.nlm.nih.gov/Blast.cgi).

Of ticks collected in Georgia, 418 *A. americanum* ticks (237 adults, 180 nymphs, and 1 unknown) and 19 *A. maculatum* ticks, were submitted for testing. Of these, 1 *A. americanum* tick (Fayette County, May 2005) and 1 *A. maculatum* tick (Morgan County, July 2005) were positive for *R. parkeri* (100% identity with GenBank accession no. U17008).

Of 611 *A. americanum* and 2 *A. maculatum* ticks collected in Tennessee, 446 *A. americanum* (164 adults and 282 nymphs) and 2 *A. maculatum* adults were individually tested for *Rickettsia* spp. An additional 103 *A. americanum* larvae were divided in 10 pools of 4–19 ticks each according to collection site. A single *A. americanum* adult male (0.2% of total) had a positive RFLP pattern that matched the previously described *rOmpA* gene pattern of *R. parkeri* (*8*). The sequence of this amplicon (532 bp, GenBank ac-

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DOI: 10.3201/eid1509.090330

cession no. FJ793521) was 99% similar to that of *R. parkeri* (EU715288). The positive tick had been collected from a coyote in Knox County, Tennessee, in July 2007.

Conclusions

We identified *R. parkeri* in ticks in Tennessee and Georgia. In Tennessee, our identification of only 2 *A. maculatum* ticks (the primary vector of *R. parkeri*) supports previous reports that this tick species is uncommon in Tennessee (*9*). In Georgia, the 19 *A. maculatum* ticks identified were <5% of the total number of ticks collected in Georgia. In contrast to these low numbers, *A. americanum* ticks are ubiquitous in high densities throughout the other southeastern states and readily feed on humans (*8*). Recently, the range of *A. americanum* ticks has expanded in the United States and now extends from west-central Texas to the Atlantic Coast, encompassing the entire Southeast and parts of the lower Midwest and coastal New England (*10*). The distribution of *A. americanum* ticks completely overlaps the suspected distribution of *A. maculatum* ticks (Figure). The positive *A. americanum* tick from Tennessee was collected from a free-ranging coyote, and the detected *R. parkeri* may have been present in the blood meal taken by the tick. However, *A. americanum* ticks can maintain and transmit *R. parkeri* infection both transovarially and transtadially (*6*). *R. parkeri* has previously been identified in *A. maculatum* ticks from Georgia (*4*), but the identification of *R. parkeri* in *A. americanum* ticks renews concerns that this tick species may be involved in the natural history of another zoonotic pathogen. Additional study is needed to determine the extent of the role of *A. americanum* ticks as a natural vector for *R. parkeri*.

This study raises concerns about the serologic diagnosis of RMSF. *R. parkeri* may be the etiologic agent of some rickettsiosis cases in Tennessee and Georgia that have been misdiagnosed as RMSF. Because reliable clinical tests specific for different SFG rickettsiae are not readily available, several different rickettsioses may be serologically cross-reactive, leading to misdiagnosis of RMSF (*11–14*). Reliable diagnosis requires PCR or culture of biopsy specimens from eschars, when present (*15*). Additional studies characterizing SFG rickettsioses, including development of rickettsial species–specific clinical tests, will assist in attributing rickettsiosis to *R. rickettsii*, *R. parkeri*, or other SFG rickettsial infections.

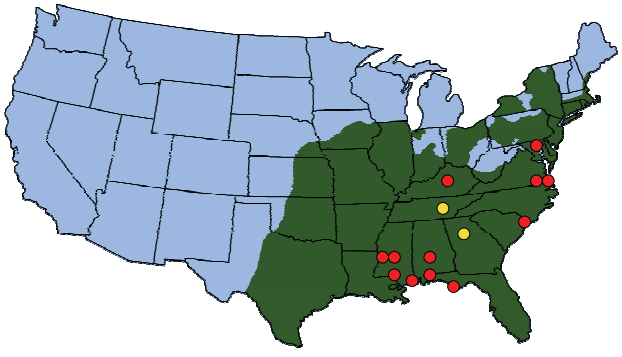

Figure. Location of ticks, *Rickettsia parkeri* in ticks, and human cases of rickettsiosis in the United States. Green shading indicates approximate distribution of *Amblyomma americanum* ticks, which completely overlaps with the known or suspected distribution of *A. maculatum.* Yellow circles indicate locations where *R. parkeri* was detected in *A. americanum* ticks (this study). Red circles indicate locations of confirmed or suspected cases of *R. parkeri* infection in humans (*11*).

This research was supported by an appointment to the Emerging Infectious Diseases Fellowship Program administered by the Association of Public Health Laboratories and funded by the Centers for Disease Control and Prevention.

Ms Cohen completed this work as part of an Emerging Infectious Diseases Laboratory Training Fellowship at the Tennessee Department of Health. She is entering a doctoral degree program at Cornell University to pursue her interests in infectious disease.

References

1. Parker RR, Hohls GM, Cox GW, David GE. Observations on an infectious agent from *Amblyomma maculatum.* Public Health Rep. 1939;54:1482–4.
2. Paddock CD, Sumner JW, Comer JA, Zaki SR, Goldsmith CS, Goddard J, et al. *Rickettsia parkeri*: a newly recognized cause of spotted fever rickettsiosis in the United States. Clin Infect Dis. 2004;38:805–11. DOI: 10.1086/381894
3. Ralph D, Pretzman C, Daugherty N, Poetter K. Genetic relationships among the members of the family *Rickettsiaceae* as shown by DNA restriction fragment polymorphism analysis. Ann N Y Acad Sci. 1990;590:541–52. DOI: 10.1111/j.1749-6632.1990.tb42264.x
4. Sumner JW, Durden LA, Goddard J, Stromdahl EY, Clark KL, Reeves WK, et al. Gulf Coast ticks (*Amblyomma maculatum*) and *Rickettsia parkeri*, United States. Emerg Infect Dis. 2007;13:751–3.
5. Goddard J. Experimental infection of lone star ticks, *Amblyomma americanum* (L.), with *Rickettsia parkeri* and exposure of guinea pigs to the agent. J Med Entomol. 2003;40:686–9.
6. Merten HA, Durden LA. A state-by-state survey of ticks recorded from humans in the United States. J Vector Ecol. 2000;25:102–13.
7. Labruna MB, Whitworth T, Bouyer DH, McBride JW, Camargo LMA, Camargo EP, et al. *Rickettsia bellii* and *Rickettsia amblyommii* in *Amblyomma* ticks from the State of Rondonia, Western Amazon, Brazil. J Med Entomol. 2004;41:1073–81.
8. Eremeeva M, Yu X, Raoult D. Differentiation among spotted fever group rickettsiae species by analysis of restriction fragment length polymorphism of PCR-amplified DNA. J Clin Microbiol. 1994;32:803–10.
9. Durden LA, Kollars TM Jr. An annotated list of the ticks (Acari: Ixodoidea) of Tennessee, with records of four exotic species for the United States. Bulletin of the Society of Vector Ecology. 1992;17:125–31.
10. Childs JE, Paddock CD. The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. Annu Rev Entomol. 2003;48:307–37. DOI: 10.1146/annurev.ento.48.091801.112728

11. Paddock CD, Finley RW, Wright CS, Robinson HN, Schrodt BJ, Lane CC, et al. *Rickettsia parkeri* rickettsiosis and its clinical distinction from Rocky Mountain spotted fever. Clin Infect Dis. 2008;47:1188–96. DOI: 10.1086/592254
12. Apperson CS, Engber B, Nicholson WL, Mead DG, Engel J, Yabsley MJ, et al. Tick-borne diseases in North Carolina: is "*Rickettsia amblyommii*" a possible cause of rickettsiosis reported as Rocky Mountain spotted fever? Vector Borne Zoonotic Dis. 2008;8:597–606.
13. Raoult D, Paddock CD. *Rickettsia parkeri* infection and other spotted fevers in the United States. N Engl J Med. 2005;353:626–7. DOI: 10.1056/NEJM200508113530617
14. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev. 2005;18:719–56. DOI: 10.1128/CMR.18.4.719-756.2005
15. Whitman TJ, Richards AL, Paddock CD, Tamminga CL, Sniezek PJ, Jiang J, et al. *Rickettsia parkeri* infection after tick bite, Virginia. Emerg Infect Dis. 2007;13:334–6.

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Phylogeny and Disease Association of Shiga Toxin–producing *Escherichia coli* O91

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The diversity and relatedness of 100 Shiga toxin–producing *Escherichia coli* O91 isolates from different patients were examined by multilocus sequence typing. We identified 10 specific sequence types (ST) and 4 distinct clonal groups. ST442 was significantly associated with hemolytic uremic syndrome.

Shiga toxin–producing *Escherichia coli* (STEC) infections are public health concerns because of the severe illnesses they cause, such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) (*1*). STEC constitute a heterogeneous group of bacteria abundant in the reservoir and in the environment (*2*). Transmission routes for human STEC infection are numerous and include contact with animal excreta, person-to-person transmission, and inadvertent ingestion of contaminated food and water. Many STEC serotypes have been recovered from humans (*3*,*4*). Among them, STEC O91 is the most common serogroup isolated from adult patients in Germany (*5*,*6*). The strains within this serogroup appear to be transmitted predominantly by food, because 1) food vehicles have been identified as the only risk factors for adults with sporadic STEC O91 infection in Germany (*6*); 2) O91 is the second most frequently isolated STEC serogroup in routine food samples (*5*); and 3) O91 is the only major STEC serogroup with no association between incidence of human infection and cattle density (*7*).

Whereas most human disease STEC serogroups possess, in addition to Shiga toxin, the *eae* gene encoding the adhesin intimin (*3*,*4*,*8*), STEC O91 consistently lack this virulence determinant (*8*,*9*). Despite frequent isolation of STEC O91 from humans, the clonal relatedness of the serotypes of this serogoup is poorly understood. Therefore, we investigated 100 human STEC O91 isolates to determine the clonal structure of STEC O91 and its association with disease.

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DOI: 10.3201/eid1509.090161

The Study

A total of 100 STEC O91 isolates were obtained from 1997 through 2007 from patients with HUS (n = 4), bloody diarrhea (n = 8), watery diarrhea without visible blood (n = 79), abdominal cramps without diarrhea (n = 1), or from asymptomatic carriers (n = 8); samples were from Germany (n = 96), Austria (n = 2; Austrian Reference Library, Innsbruck, Austria), Finland (n = 1; The National Public Health Institute, Helsinki, Finland), and Canada (n = 1; Public Health Agency of Canada, Guelph, Ontario, Canada). The 96 German O91 strains were recovered at the Institute of Hygiene, University of Münster, Münster, and the Robert Koch Institute, Wernigerode, Germany. The strains included all human isolates of this serogroup that were recovered during the study period in Germany and for which complete clinical information was available. The strains correspond to all O91 serotypes associated with human diseases from sporadic cases in Germany in that interval. Thirty-five strains have been described previously (*4*,*8*,*10*).

The age of patients from whom the STEC O91strains originated ranged from 4 months to 89 years (median 28 years, interquartile range 12–38 years). The most severe symptom was recorded for each patient. Diarrhea was defined as ≥3 semisolid or liquid stools per day. Bloody diarrhea was defined as diarrheal stools containing blood visible to the naked eye. HUS was defined as a case of microangiopathic hemolytic anemia (hematocrit <30% with peripheral evidence of intravascular hemolysis), thrombocytopenia (platelet count <150,000/mm^3), and renal insufficiency (serum creatinine concentration greater than the upper limit of normal for age) (*11*). Asymptomatic carriers were apparently healthy persons without diarrhea; their stools were submitted as noted above.

Strains were isolated using Shiga toxin–encoding genes as diagnostic targets (*12*) and then serotyped phenotypically (*13*). All strains were verified as O91 by using PCR targeting wzy_{O91}, a component of the *rfb* gene cluster that synthesizes the O91 antigen (*14*). Multilocus sequence typing (MLST) and phylogenetic analysis were performed as described (*4*). All allelic sequences were deposited in the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli). The minimum spanning tree was generated from all 100 O91 sequence types (STs) and compared with the HUS-associated enterohemorrhagic *E. coli* (HUSEC) collection (*4*) to display the distribution of the STs compared with all known STs associated with HUS.

We tabulated disease severity according to STs. To study the relationship between ST442 and disease severity, patients were categorized into those with HUS, those with bloody diarrhea, and, serving as reference group, those with nonbloody diarrhea or those that asymptomatically excreted these organisms. Univariate associations were computed by using exact logistic regression. p values <0.05 were considered statistically significant. STATA release 10.0 (StataCorp LP, College Station, TX, USA) was used for statistical analysis.

The 100 STEC O91 strains resulted in 10 different STs. Of these, STs 33 and 442 were most common (63 and 20 isolates, respectively). Six additional STs (690, 1048, 1051, 1052, 1053, 1054) were single-locus variants of ST33, indicating their close relationship. The 2 remaining STs (641 and 1020) were not closely related to any other ST of the serogroup O91 strains. Detailed analysis of the 7 housekeeping genes used for MLST demonstrated the *fumC* gene sequence alone could differentiate 5 STs of the O91 strains. The comparison of all O91 STs with all STEC STs and serotypes associated with HUS (HUSEC collection) is displayed in the Figure.

H antigens associated with O91 were H8 (n = 1), H10 (n = 2), H14 (n = 52), H21 (n = 20), Hnt (H-antigen nontypeable) (n = 10), and H$^-$ (H-antigen nonmotile) (n = 15). In serotype O91:H8, O91:H10, and O91:H21 strains, the STs were serotype-specific. However, ST33 and its single-locus variants represented all strains of serotypes O91:H14, O91: H$^-$, and O91:Hnt.

A single sequence type, ST442, accounting for 20% of all strains, was found among each of the four O91 isolates from patients with HUS (Table 1), a highly significant association (odds ratio [OR] 27.8, 95% confidence interval [CI] 3.3–∞, p<0.01; Table 2). ST442 strains were also more frequently isolated from patients with bloody diarrhea than were strains belonging to other STs (3/20 [15.0%] vs. 5/80 [6.3%] respectively), but this difference was not statistically significant (Table 2). The overall association with

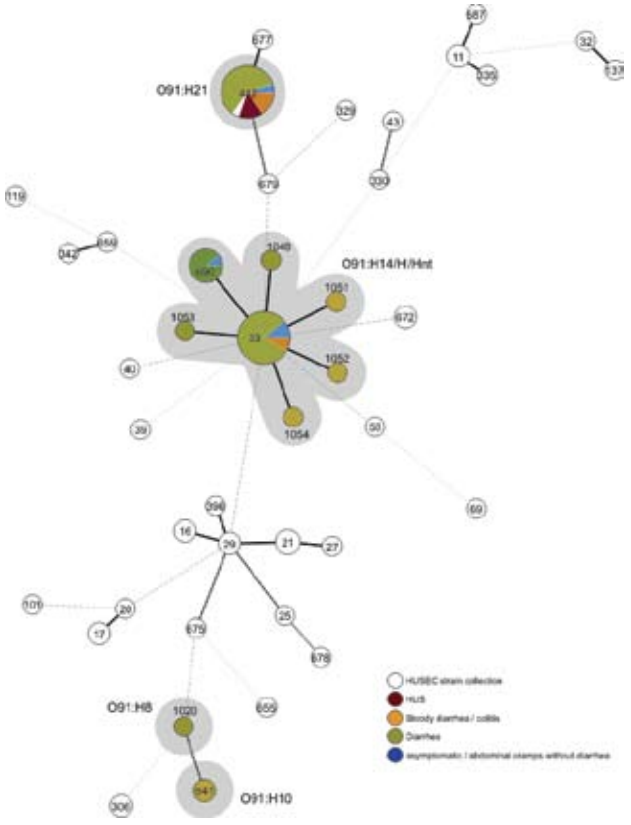

Figure. Minimum spanning tree based on the multilocus sequence typing allelic profiles portraying the clonal distribution of the 100 *Escherichia coli* O91:H8/H10/H14/H21/H$^-$/Hnt isolates (highlighted in gray) associated with different diseases in relation to the hemolytic uremic syndrome–associated enterohemorrhagic *E. coli* collection. Each dot represents a given sequence type, and the size of each circle is proportional to the number of strains analyzed. Connecting lines show the number of identical alleles between 2 STs (thick black line, 6 of 7 alleles identical; thick gray line, 5 alleles identical; thick dashed line, 4 alleles identical; thin dashed lines of increasing length, ≤3 alleles identical).

Table 1. Distribution of disease severity across 10 different sequence types of 100 STEC O91 strains isolated from humans*

ST (Serotype)	Most severe symptom of patients infected with STEC O91				Total no. strains
	HUS (n = 4)	BD (n = 8)	D (n = 79)	A (n = 9)	
ST33 (O91:H14/H$^-$/Hnt)	0	5	51	7	63
ST442 (O91:H21)	4	3	12	1†	20
ST641 (O91:H10)	0	0	2	0	2
ST690 (O91:H14)	0	0	8	1	9
ST1020 (O91:H8)	0	0	1	0	1
ST1048 (O91:H14)	0	0	1	0	1
ST1051 (O91:H14)	0	0	1	0	1
ST1052 (O91:H14)	0	0	1	0	1
ST1053 (O91:H14)	0	0	1	0	1
ST1054 (O91:Hnt)	0	0	1	0	1

*ST, sequence type; STEC, Shiga toxin–producing *Escherichia coli*; H, H-antigen nonmotile strains; Hnt, H-antigen nontypeable strains; HUS, hemolytic uremic syndrome; BD, bloody diarrhea; D, diarrhea without visible blood; A, asymptomatic infection (1 patient infected with ST442 [STEC O91:H21] had abdominal cramps without diarrhea).
†Abdominal cramps without diarrhea.

Table 2. Univariate associations of STEC O91 of sequence type 442 with severe disease by use of exact logistic regression*

Severe disease	ST442, no. (%)	Non-ST442, no. (%)	Odds ratio	95% Confidence interval	p value
HUS	4 (20)	0 (0)	27.8†	3.29–∞	<0.01
BD	3 (15)	5 (6)	3.4	0.47–20.1	0.25
HUS or BD	7 (35)	5 (6)	7.8	1.83–36.6	<0.01

*Reference group consisted of persons who had nonbloody diarrhea or who were asymptomatic carriers. STEC, Shiga toxin-producing *Escherichia coli*; ST, sequence type; HUS, hemolytic uremic syndrome; BD, bloody diarrhea.
†Mean unbiased estimate.

severe disease, defined as either HUS or bloody diarrhea, was strong (OR 7.8, 95% CI 1.83–36.6, p<0.01). Severe illness was noted for 7 (35.0%) of 20 patients infected by ST442 strains, but only for 5 (6.3%) of 80 patients infected by STEC O91 of other STs (Table 2). Patients with bloody diarrhea were younger (median age 12 years) than patients who had mild or no symptoms (median age 20 years). However, this difference was not observed for the 4 patients with HUS (median age 21 years); in this instance, 2 were adults, 1 was 39 months old, and 1 was unknown.

Conclusions

To gain insight into the clonal structure of STEC O91, we determined the relatedness of 100 strains isolated from patients and correlated the clonal lineage to the clinical outcome of the infection. MLST analysis divided the O91 isolates into 10 different STs, whereas classical serotyping identified only 4 complete serotypes (O- and H-antigen). Moreover, MLST was able to type all 25 nonmotile (H^-) or nontypeable (Hnt) O91 strains. The analysis demonstrated that the *fumC* gene from the 7 genes used for MLST was the most heterogeneous and enabled strain differentiation into 5 different STs, among these ST442. It might therefore be a candidate for first-line single-locus sequence typing.

HUS or bloody diarrhea without HUS was significantly associated with ST442, which was represented by serotype O91:H21 only. However, Pradel et al. also reported a case of HUS associated with an O91:H10 isolate that could be differentiated from O91:H21 by using ribotyping (*15*). In our study, known virulence determinants such as cytolethal distending toxin V or Shiga toxin 2d activatable by elastase in O91:H21 strains (*8*,*10*) might contribute to the higher virulence of O91:H21 (ST442). However, further studies of the mechanisms behind the emergence of ST442 in Germany and additional analysis of global O91 isolates are needed. With the MLST approach described, trends and changes in STEC O91 epidemiology and human infections can be carefully surveyed.

Acknowledgments

We thank M. Karmali, D. Orth, and A. Siitonen for kindly providing O91 strains from their countries. We are also grateful to P. Tarr for critical reading of the manuscript.

This study was supported by grants from the Federal Ministry of Education and Research Network Zoonoses (grant nos. 01KI07124, 01KI07120, 01KI07127, 1368-763) and from the National Reference Laboratory Network of the Robert Koch Institute.

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References

1. Karch H, Tarr PI, Bielaszewska M. Enterohaemorrhagic *Escherichia coli* in human medicine. Int J Med Microbiol. 2005;295:405–18. DOI: 10.1016/j.ijmm.2005.06.009
2. Caprioli A, Morabito S, Brugere H, Oswald E. Enterohaemorrhagic *Escherichia coli:* emerging issues on virulence and modes of transmission. Vet Res. 2005;36:289–311. DOI: 10.1051/vetres:2005002
3. Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, et al. Non-O157 Shiga toxin–producing *Escherichia coli* infections in the United States, 1983–2002. J Infect Dis. 2005;192:1422–9. DOI: 10.1086/466536
4. Mellmann A, Bielaszewska M, Köck R, Friedrich AW, Fruth A, Middendorf B, et al. Analysis of collection of hemolytic uremic syndrome–associated enterohemorrhagic *Escherichia coli.* Emerg Infect Dis. 2008;14:1287–90. DOI: 10.3201/eid1408.071082
5. Werber D, Beutin L, Pichner R, Stark K, Fruth A. Shiga toxin–producing *Escherichia coli* serogroups in food and patients, Germany. Emerg Infect Dis. 2008;14:1803–6. DOI: 10.3201/eid1411.080361
6. Werber D, Behnke SC, Fruth A, Merle R, Menzler S, Glaser S, et al. Shiga toxin–producing *Escherichia coli* infection in Germany: different risk factors for different age groups. Am J Epidemiol. 2007;165:425–34. DOI: 10.1093/aje/kwk023
7. Frank C, Kapfhammer S, Werber D, Stark K, Held L. Cattle density and Shiga toxin–producing *Escherichia coli* infection in Germany: increased risk for most but not all serogroups. Vector Borne Zoonotic Dis. 2008;8:635–43. DOI: 10.1089/vbz.2007.0237
8. Bielaszewska M, Friedrich AW, Aldick T, Schurk-Bulgrin R, Karch H. Shiga toxin activatable by intestinal mucus in *Escherichia coli* isolated from humans: predictor for a severe clinical outcome. Clin Infect Dis. 2006;43:1160–7. DOI: 10.1086/508195
9. Bettelheim KA. The non-O157 Shiga-toxigenic (verocytotoxigenic) *Escherichia coli;* under-rated pathogens. Crit Rev Microbiol. 2007;33:67–87. DOI: 10.1080/10408410601172172
10. Bielaszewska M, Fell M, Greune L, Prager R, Fruth A, Tschäpe H, et al. Characterization of cytolethal distending toxin genes and expression in Shiga toxin–producing *Escherichia coli* strains of non-O157 serogroups. Infect Immun. 2004;72:1812–6. DOI: 10.1128/IAI.72.3.1812-1816.2004
11. Tarr PI, Gordon CA, Chandler WL. Shiga toxin–producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet. 2005;365:1073–86.
12. Mellmann A, Bielaszewska M, Zimmerhackl LB, Prager R, Harmsen D, Tschäpe H, et al. Enterohemorrhagic *Escherichia coli* in human infection: in vivo evolution of a bacterial pathogen. Clin Infect Dis. 2005;41:785–92. DOI: 10.1086/432722

13. Prager R, Strutz U, Fruth A, Tschäpe H. Subtyping of pathogenic *Escherichia coli* strains using flagellar (H)-antigens: serotyping versus *fliC* polymorphisms. Int J Med Microbiol. 2003;292:477–86. DOI: 10.1078/1438-4221-00226
14. Perelle S, Dilasser F, Grout J, Fach P. Identification of the O-antigen biosynthesis genes of *Escherichia coli* O91 and development of a O91 PCR serotyping test. J Appl Microbiol. 2002;93:758–64. DOI: 10.1046/j.1365-2672.2002.01743.x
15. Pradel N, Boukhors K, Bertin Y, Forestier C, Martin C, Livrelli V. Heterogeneity of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic-uremic syndrome patients, cattle, and food samples in central France. Appl Environ Microbiol. 2001;67:2460–8. DOI: 10.1128/AEM.67.6.2460-2468.2001

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Human *Plasmodium knowlesi* Infection Detected by Rapid Diagnostic Tests for Malaria

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We describe a PCR-confirmed case of *Plasmodium knowlesi* infection with a high parasitemia level and clinical signs of severe malaria in a migrant worker from Malaysian Borneo in the Netherlands. Investigations showed that commercially available rapid antigen tests for detection of human *Plasmodium* infections can detect *P. knowlesi* infections in humans.

The malaria parasite *Plasmodium knowlesi* naturally occurs in long-tailed and pig-tailed macaques that inhabit forested areas in Southeast Asia. *P. knowlesi* can be transmitted from monkeys to humans by the bite of an infected mosquito (*1*), but infection with *P. knowlesi* was traditionally regarded as a rare disease, occurring only sporadically in humans. However, recent findings of a large number of infected patients in Malaysian Borneo; other reports of human cases in Thailand, Myanmar, the Philippines, and Singapore; and some reports of *P. knowlesi* malaria acquired by travelers to the Malaysian Borneo suggest that *P. knowlesi* may be more widespread among humans than previously thought (*2*–*7*).

Unfortunately, microscopic analysis of asexual stages of *P. knowlesi* can misidentify these parasites as *P. malariae* (*3*,*4*). Unlike *P. malariae*, which multiplies every 3 days in the blood and never results in severe infections, *P. knowlesi* multiplies daily, and high parasitemia with death in humans can occur (*4*). Therefore, early diagnosis and immediate treatment is warranted. Although PCR and sequencing are used in species confirmation, a more rapid diagnostic test would be a useful tool for delivering prompt and adequate medical treatment. We report a case of imported *P. knowlesi* infection in the Netherlands in a migrant worker from Malaysian Borneo. This *P. knowlesi* infection was detected by commercially available rapid diagnostic antigen tests for malaria.

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DOI: 10.3201/eid1509.090358

The Case

A 38-year-old man came to the Netherlands in January 2009 to work as a rigger in the harbor of Rotterdam. Since October 2008, he had lived in Kapit, Sarawak, in Borneo and hunted wild animals in the surrounding jungles. One week after arriving in the Netherlands, he came to our hospital with a 5-day history of fever, myalgia, headache, and low back pain. His medical history was uneventful, and he had not experienced any previous malaria attacks.

Physical examination showed a temperature of 40.0°C and remarkable jaundice. Abdominal examination showed no abnormalities. Laboratory investigations showed a moderate anemia (hemoglobin 7.8 mmol/L [reference range 8.5–11.0 mmol/L]), a normal leukocyte count (5.8×10^9/L [reference range $4.3–10 \times 10^9$/L]), thrombocytopenia (platelet count 22×10^9/L [reference range $150–400 \times 10^9$/L]), an increased level of C-reactive protein (158 mg/L [reference range <10 mg/L]), and liver function abnormalities (serum alanine aminotransferase 199 U/L [reference range <41 U/L]; aspartate aminotransferase 128 U/L [reference range <37 U/L]; lactate dehydrogenase [LDH] 1,059 U/L [reference range <450 U/L]; gamma-glutamyltransferase 183 U/L [reference range <50 U/L]; alkaline phosphatase 285 U/L [reference range <120 U/L]; and total bilirubin 99 μmol/L [reference range <17 μmol/L]). Plasma lactate level was within normal limits.

In a rapid diagnostic test for malaria (BinaxNOW Malaria Test; Binax, Inc., Scarborough, ME, USA), his blood sample was negative for *P. falciparum* histidine-rich protein 2 but showed a positive reaction with pan-malarial aldolase antigen, which suggested a non–*P. falciparum* infection. Results of quantitative buffy coat analysis were positive for malaria trophozoites, schizonts, and gametocytes. A thin blood film showed parasite density of 2% infected erythrocytes (84,000 trophozoites/μL), schizonts, and gametocytes with an inconclusive morphologic appearance (Figure 1). A *P. knowlesi* infection was suspected because of his recent stay in Kapit, Malaysian Borneo. The patient was treated orally with chloroquine, 10 mg/kg, followed by 5 mg/kg after 6, 24, and 48 hours, which resulted in a rapid relief of symptoms and fever. Results of quantitative buffy coat analysis, pan-malarial aldolase antigen reactivity, and thick and thin blood smears were negative within 40 hours after administration of chloroquine.

Subsequently, PCR analysis of blood samples taken at admission was performed to determine the *Plasmodium*

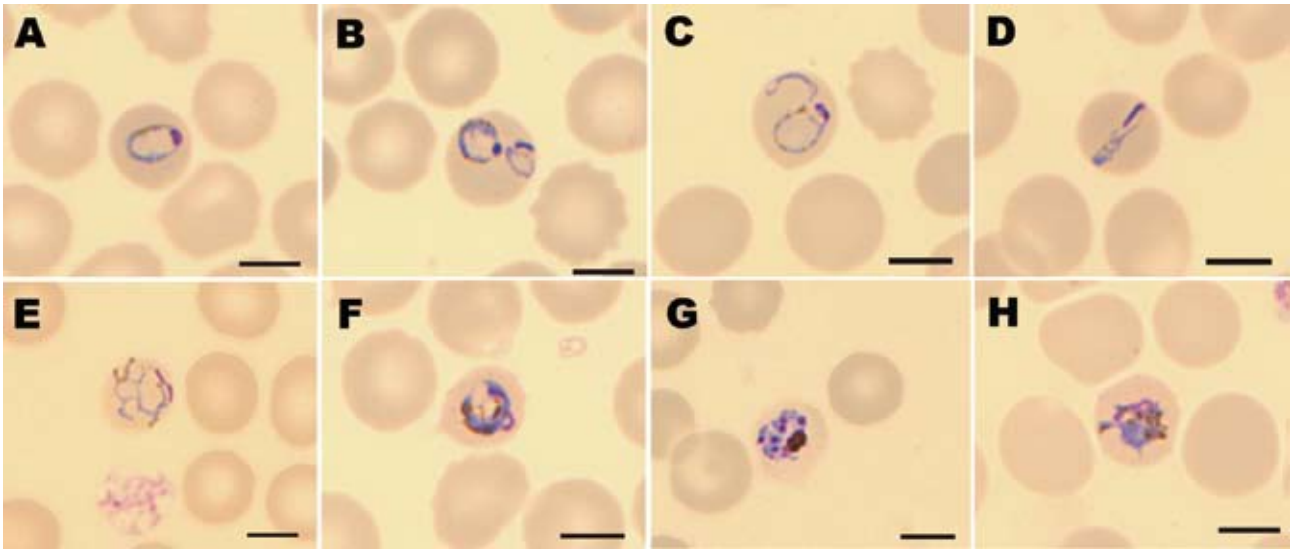

Figure 1. Morphology of *Plasmodium knowlesi* in a Giemsa-stained thin blood smear. Infected erythrocytes were not enlarged, lacked Schuffner stippling, and contained much pigment. Shown are examples of trophozoites (A–F), a schizont (G), and a gametocyte (H). Scale bars = 5 μm.

species. Human *Plasmodium* species were excluded by using a conventional nested PCR and real-time PCR (*8*,*9*). In addition, PCR analysis was performed on a blood sample by using diagnostic primers for *Plasmodium* small subunit (SSU) rRNA as described (*3*), including genus-specific and species-specific primers. In contrast to the method described by Singh et al. (*3*), nested PCR was not necessary because of high parasitemia and availability of fresh material. Instead, PCRs were performed directly on 2 μL of blood in 25-μL volumes by using the Phusion Blood PCR kit (Finnzymes, Espoo, Finland). Genus-specific primer sets and *P. knowlesi*–specific primers generated PCR products, providing evidence that the patient had *P. knowlesi* malaria.

To confirm the PCR result, we sequenced the cloned amplification product generated with primers rPLU1 and rPLU5. Sequences were compared with known *Plasmodium* A-type SSU rRNA sequences by using the neighbor-joining method (Figure 2). The sequence of the clinical isolate PkHHR-BPRC1 (GenBank accession no. FJ804768) clustered strongly with *P. knowlesi* A-type SSU RNA sequences, confirming that the patient was infected with the *P. knowlesi* parasite.

Conclusions

Imported malaria is relatively rare in industrialized countries. Obtaining a correct diagnosis of malaria may be troublesome in centers where laboratory staff are less skilled in the proper identification and quantification of causative *Plasmodium* species, as may occur in countries in which malaria is not endemic. These centers often use commercially available rapid diagnostic tests to diagnose malaria. In contrast to our case, Bronner et al. reported that the BinaxNOW Malaria Test did not detect a *P. knowlesi* infection in a traveler from Sweden who had a *P. knowlesi* infection acquired in Malaysian Borneo (*2*). Low parasitemia (0.1%) in this patient may have caused the lack of reactivity with the pan-malarial antigen aldolase (*2*).

We evaluated the BinaxNOW Malaria and the OptiMAL Rapid Malaria (Diamed, Cressier, Switzerland)

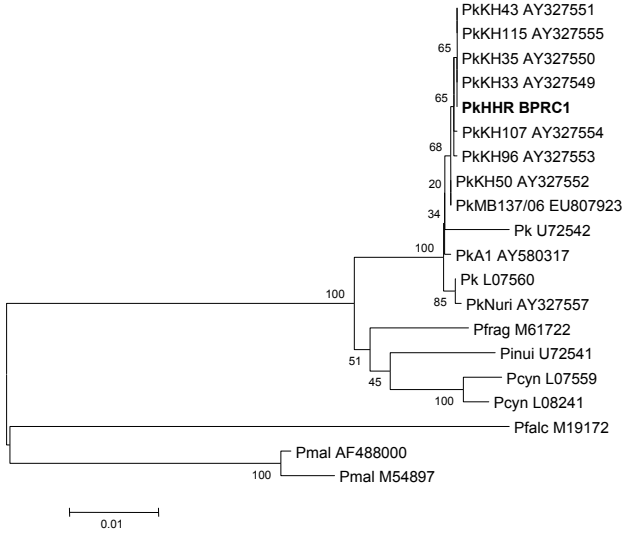

Figure 2. Phylogenetic tree constructed according to the neighbor-joining method based on A-type small subunit RNA sequences of several *Plasmodium* species (GenBank accession numbers are indicated). The sequence of the clinical isolate PkHHR-BPRC1 (in **boldface**) (GenBank accession no. FJ804768) clusters with all other *P. knowlesi* strains (indicated by Pk isolate numbers). Pfrag, *P. fragile*; Pinui, *P. inui*; Pcyn, *P. cynomolgi;* Pfalc, *P. falciparum;* Pmal, *P. malariae*. Scale bar indicates nucleotide substitutions per site.

Table. Evaluation of 2 commercially available rapid diagnostic antigen tests used for detection of *Plasmodium knowlesi**

Time after admission, h	*P. knowlesi* parasitemia, trophozoites/μL	BinaxNOW†		Diamed OptiMAL‡	
		HRP-2	Aldolase	*P. falciparum*–LDH	Pan-malarial LDH
0	84,000	–§	+§	+	+
16	1,587	–§	–§	+	+
24	138	–	–	–	–
40	ND	–	–	–	–

*HRP-2, histidine-rich protein 2; LDH, lactate dehydrogenase; ND, not detectable. All tests were preformed on blood samples collected in EDTA and frozen at –20°C for 2 weeks.
†Binax, Inc., Scarborough, ME, USA
‡Diamed, Cressier, Switzerland.
§Tests were also performed on freshly collected blood samples; results were identical.

tests for detection of *P. knowlesi* in human blood by analysis of consecutive blood samples taken after admission. These samples were stored at –20°C for 2 weeks until tests were performed (Table). The blood sample taken on admission (2% infected erythrocytes) did not react with *P. falciparum*–specific antibody against histidine-rich protein 2 but reacted with the pan-malarial antigen aldolase in the BinaxNOW Malaria Test. This sample also showed a positive result in the *P. falciparum*–specific LDH and pan-malarial LDH in the OptiMAL Rapid Malaria Test, confirming the cross-reactivity of *P. knowlesi* LDH with monoclonal antibody 17E4 against *P. falciparum* LDH, as shown by McCutchan et al. (*10*). This antibody is also used in the OptiMAL Rapid Malaria Test (Diamed, pers. comm.). Therefore, a positive test result for the *P. falciparum* LDH in the OptiMAL Rapid Malaria Test is not specific for *P. falciparum* because it can also be caused by a *P. knowlesi* infection. The positive result for LDH and aldolase in either test became negative after treatment (Table), which indicates rapid clearance of parasites after treatment. Results of our comparative study suggest that the OptiMAL Rapid Malaria Test may be able to detect lower levels of *P. knowlesi* parasitemia than the BinaxNOW Malaria Test.

Our results indicate that commercially available rapid diagnostic antigen tests for human *Plasmodium* species can detect *P. knowlesi* infections in humans, although infections with a low parasitemia will not be detected. A negative test result does not exclude a *P. knowlesi* infection, as it does not exclude infections by other human *Plasmodium* species (*11*).

Acknowledgments

We thank Ton van den Berg for preparing blood films and detecting the *P. knowlesi* infection and Ernst Verschoor for helping with phylogenetic analysis of SSU rRNA sequences.

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References

1. Chin W, Contacos PG, Collins WE, Jeter MH, Alpert E. Experimental mosquito transmission of *Plasmodium knowlesi* to man and monkey. Am J Trop Med Hyg. 1968;17:355–8.
2. Bronner U, Divis PC, Farnert A, Singh B. Swedish traveller with *Plasmodium knowlesi* malaria after visiting Malaysian Borneo. Malar J. 2009;8:15. DOI: 10.1186/1475-2875-8-15
3. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet. 2004;363:1017–24. DOI: 10.1016/S0140-6736(04)15836-4
4. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratman S, et al. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. Clin Infect Dis. 2008;46:165–71. DOI: 10.1086/524888
5. Kantele A, Marti H, Felger I, Muller D, Jokiranta TS. Monkey malaria in a European traveler returning from Malaysia. Emerg Infect Dis. 2008;14:1434–6. DOI: 10.3201/eid1409.080170
6. Ng OT, Ooi EE, Lee CC, Lee PJ, Ng LC, Pei SW, et al. Naturally acquired human *Plasmodium knowlesi* infection, Singapore. Emerg Infect Dis. 2008;14:814–6. DOI: 10.3201/eid1405.070863
7. Jongwutiwes S, Putaporntip C, Iwasaki T, Sata T, Kanbara H. Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. Emerg Infect Dis. 2004;10:2211–3.
8. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol Biochem Parasitol. 1993;58:283–92. DOI: 10.1016/0166-6851(93)90050-8
9. Muller-Stover I, Verweij JJ, Hoppenheit B, Gobels K, Haussinger D, Richter J. *Plasmodium malariae* infection in spite of previous anti-malarial medication. Parasitol Res. 2008;102:547–50. DOI: 10.1007/s00436-007-0804-4
10. McCutchan TF, Piper RC, Makler MT. Use of malaria rapid diagnostic test to identify *Plasmodium knowlesi* infection. Emerg Infect Dis. 2008;14:1750–2.
11. van den Broek I, Hill O, Gordillo F, Angarita B, Hamade P, Counihan H, et al. Evaluation of three rapid tests for diagnosis of *P. falciparum* and *P. vivax* malaria in Colombia. Am J Trop Med Hyg. 2006;75:1209–15.

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Monitoring of Putative Vectors of Bluetongue Virus Serotype 8, Germany

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To identify the vectors of bluetongue virus (BTV) in Germany, we monitored *Culicoides* spp. biting midges during April 2007–May 2008. Molecular characterization of batches of midges that tested positive for BTV suggests *C. obsoletus* sensu stricto as a relevant vector of bluetongue disease in central Europe.

Bluetongue disease (BT), discovered north of the Alps in Europe in August 2006 (*1*–*5*), causes massive losses of farm ruminants, particularly sheep. Epidemic BT has been caused by serotype 8 of bluetongue virus (BTV-8). The virus overwintered and spread over a large area in 2007 (*5*,*6*). *Culicoides* spp. biting midges can transmit BT. In the Mediterranean region, BT is mainly transmitted by *C. imicola* midges, a species that has so far not been detected north of the Alps (*7*,*8*). We aimed to determine the abundance of hematophagous *Culicoides* spp. biting midges and to identify putative vectors of BTV in Germany.

The Study

Biting midges were caught from April 2007 through May 2008 by using 89 black light traps (BG-Sentinel Trap; Biogents, Regensburg, Germany) distributed mostly in the German BT restriction zone of January 2007 (Figure 1). Most (n = 85) traps were placed in the vicinity of cow sheds, either adjacent to barns or in their entrance area; 4 additional traps were placed in pastures if the cattle were kept there day and night.

Biting midges were caught during 7 consecutive nights in the first week of each month. We preserved them in 70% ethanol and sorted them under the dissection microscope according to wing patterns (*9*,*10*) into batches of <50 parous, nulliparous, or blood-fed female or male insects of the *C. obsoletus* group (including *C. dewulfi*), the *C. pulicaris* group, or other *Culicoides* spp. biting midges.

Batches of female biting midges were tested for BTV by real-time reverse transcription–PCR (rRT-PCR). Each batch was homogenized in 400 μL lysis buffer (NucleoSpin 96 Virus kit; Macherey-Nagel, Düren, Germany) with a 5-mm steel bead by using a TissueLyser instrument (QIAGEN, Hilden, Germany) for 2 min at 30 Hz. After short centrifugation at 12,000 × *g*, nucleic acids were extracted from 200 μL homogenate (NucleoSpin 96 Virus kit) on a Tecan Freedom EVO automatic platform (Tecan Deutschland GmbH, Crailsheim, Germany). RNA was ana-

Figure 1. *Culicoides* spp. monitoring area, 150-km zone restricted because of the occurrence of bluetongue disease in Germany as of January 2007 (blue border), and geographic positions of 89 black light traps (red dots).

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DOI: 10.3201/eid1509.090562

lyzed by using the iScript One-Step RT-PCR Kit for Probes (Bio-Rad, Munich, Germany) in a duplex rRT-PCR (pan-BTV-duplex rRT-PCR) combining a BTV rRT-PCR that detects all known serotypes (*11*) with a PCR that detects all members of the genus *Culicoides* (pan-*Culicoides* assay) as an internal control for RNA extraction and amplification. For the pan-*Culicoides* assay, primers and a probe were selected from aligned sequences of the rDNA internal transcribed spacer 1 and 2 (Pan-Culi-ITS1+2-597F [5′-CAG GAC ACA CGA TCA TTG ACA-3′], Pan-Culi-ITS1+2-976R [5′-CAC ATG AGY TGA GGT CGT CAT-3′], Pan-Culi-ITS1+2-623HEX [5′-HEX-AAC GCA TAT TGC ACC CCA TGC GA-BHQ1-3′]). To confirm positive pan-BTV-duplex rRT-PCR results, we extracted RNA from the remaining homogenate of the batch and subjected it to BTV-8- rRT-PCR (*5*). Batches were considered BTV positive if results of both assays were positive. Batches of the *C. obsoletus* and *C. pulicaris* groups with high viral loads were further analyzed for *Culicoides* spp. by amplification of the mitochondrial cytochrome oxidase subunit I (*12*).

The overall number of biting midges caught started at a moderate level (11,577) in April 2007, peaked in October (246,882), decreased to low levels during December 2007–March 2008, and started to rise again (462) in April 2008 (Figure 2). Small numbers (66–81) of *Culicoides* spp. midges also were detected in some traps during January–March 2008. Members of the *C. obsoletus* group (including *C. dewulfi*) were most frequently trapped, followed by the *C. pulicaris* group. Biting midges of the *C. pulicaris* group were more often collected during spring and summer 2007 in discrete locations.

Of 24,513 batches analyzed by rRT-PCR, 16,206 (66.1%) batches belonged to the *C. obsoletus* group, 5,796 (23.6%), to the *C. pulicaris* group, and 2,511 (10.2%) to other *Culicoides* spp. A total of 585 (2.4%) batches were positive for BTV by rRT-PCR, 562 (96.1%) of which belonged to the *C. obsoletus* group, 16 (2.7%), to the *C. pulicaris* group, and 6 (1.0%) to other *Culicoides* spp.; 1 was identified as *C. achrayi*. The number of positive pools varied considerably by month (Figure 2). All batches that were positive in the pan-BTV-rRT-PCR analysis with a cycle threshold (Ct) value <37 (n = 464) were confirmed as BTV positive. BTV-infected biting midges (*C. pulicaris* group) were first detected in June 2007, a few weeks after the first new infection of the year with BTV-8 had been discovered (*5*). The number of BTV-positive *Culicoides* batches peaked (n = 401) in October 2007, which coincided with the peak of midge abundance (Figure 2). During December 2007–May 2008, no BTV-positive batches were detected.

A total of 540 batches of biting midges carried a low or medium (Ct values >30–40), 38 a high (Ct values 25–30), and 7 a very high (Ct values <25) BTV genome load. Batches with a high virus genome load showed Ct values

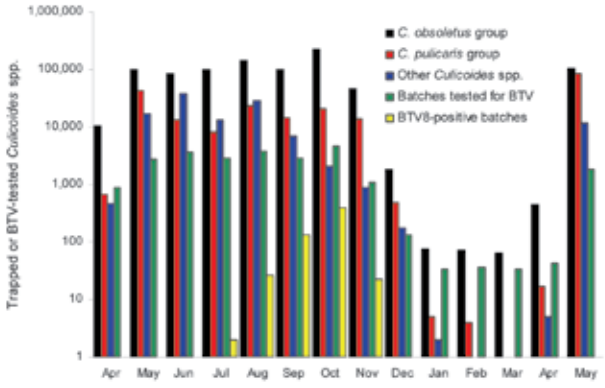

Figure 2. Monthly catches of midges of the *Culicoides obsoletus* group (black), *C. pulicaris* group (red), and other *Culicoides* spp. (blue) captured with 89 black light traps in Germany during 7 consecutive nights in the first week of each month during the study period (April 2007–May 2008). Batches consisting of ≤50 female biting midges were tested for bluetongue virus (BTV) by real-time reverse transcription–PCR. The total number of batches (green) and the number of batches positive for BTV (gold) are shown.

similar to that of highly positive, undiluted blood samples from cattle or sheep. Although ≈70–100 μL of cattle or sheep blood are used for the BTV genome detection, <1 μL blood remains in a biting midge after a blood meal. The uptake of highly BTV-positive blood can therefore only lead to a Ct value increased at least by 6 or 7 when the biting midge is tested by rRT PCR for BTV. Our findings provide strong evidence for virus replication in the biting midges in the highly positive pools.

All batches with very high and 36 batches with a high virus genome load consisted of midges of the *C. obsoletus* group. Only 2 batches with a high viral load belonged to the *C. pulicaris* group. These data clearly support the previously suggested role of species of the *C. obsoletus* group as competent vectors for BTV (*13*,*14*).

The species composition of batches of the *C. obsoletus* and *C. pulicaris* groups with a high viral genome load was further determined by PCRs of the mitochondrial cytochrome oxidase subunit I (*10*). Although our analyses showed that most batches consisted of several species (Table 1), *C. obsoletus* sensu stricto was identified in all investigated batches of the *C. obsoletus* group (Table 1). Furthermore, 18 BTV-positive batches morphologically classified as *C. obsoletus* group consisted exclusively of *C. obsoletus* sensu stricto. These findings indicate that *C. obsoletus* sensu stricto is involved in the transmission of BTV in Germany, but a role for other members of the *C. obsoletus* group in the transmission of BTV cannot be ruled out. In contrast, the characterization of the pools of the *C. pulicaris* group yielded inconclusive results (Table 2). More than 1 species-specific fragment was amplified in

Table 1. Genetic characterization of batches of midges of the *Culicoides obsoletus* group, Germany, April 2007–May 2008*

Batch no.	rRT-PCR, Ct	*C. obsoletus sensu stricto*	*C. scoticus*	*C. chiopterus*	*C. dewulfi*
270/38F	20.10	+++	–	+++	+++
276/2T	20.74	+++	–	+++	+++
306/12FN	21.33	+++	–	+++	+++
304/34B	21.60	+++	–	+++	–
276/2O	23.40	+++	–	+++	+++
296/52Q	23.87	+++	–	+++	+++
296/44G	24.00	+++	–	+++	+++
296/22R	25.90	+++	–	+++	+++
296/52AL	27.26	+++	–	+++	+++
306/1X	27.30	+++	–	–	–
306/1A	27.60	+++	–	–	–
306/1E	27.70	+++	–	–	–
306/1Z	27.70	+++	–	–	–
270/45B	27.76	+++	–	–	+++
306/1L	27.90	+++	–	–	–
306/1T	27.90	+++	–	–	–
263/46B	28.00	+++	–	++	+++
306/1H	28.00	+++	–	–	–
306/1AA	28.00	+++	–	–	–
306/1S	28.10	+++	–	–	–
306/1AC	28.10	+++	–	–	–
18/156B	28.18	+++	–	+	+++
346/29C	28.19	+++	–	–	+
306/1R	28.30	+++	–	–	–
306/1V	28.30	+++	–	–	+++
306/1N	28.40	+++	++	–	–
276/10S	28.41	+++	–	+++	+++
306/1I	28.50	+++	–	–	–
306/1M	28.70	+++	+	+++	–
263/46A	28.80	+++	–	+++	–
296/51AH	28.82	+++	++	+++	+++
306/1D	28.90	+++	–	–	–
171/13E	29.00	+++	–	–	+++
306/1C	29.00	+++	+++	–	–
304/1H	29.10	+++		+++	+++
306/1B	29.20	+++	–	–	–
306/1F	29.20	+++	–	–	–
306/1U	29.20	+++	–	–	–
336/24B	29.21	+++	–	+++	–
306/12LR	29.26	+++	–	+++	+++
306/1K	29.30	+++	–	–	–
306/1J	29.40	+++	–	–	–
270/55I	29.47	+++	–	+++	+++

*rRT-PCR, real-time reverse transcription–PCR; Ct, cycle threshold; –, no visible band; +, faint; ++, distinct; +++, strong band after agarose gel electrophoresis of PCR products and staining with ethidium bromide.

Table 2. Genetic characterization of batches of midges of the *Culicoides pulicaris* group, Germany, April 2007–May 2008*

Batch no.	rRT-PCR, Ct	*C. pulicaris* s.s.	*C. punctatus*	*C. impunctatus*	*C. grisescens*	*C. newsteadi*
263/49 A	28.57	–	++	+	+	–
263/49 B	29.46	+++	+++	++	+	+
276/46B	31.37	–	+++	–	+	+++
276/62A	34.43	+++	+++	–	–	–
292/4A	33.97	+++	+++	–	–	–
304/63A	32.90	+++	+++	+	–	++

*rRT-PCR, real-time reverse transcription–PCR; Ct, cycle threshold; –, no visible band; +, faint; ++, distinct; +++, strong band after agarose gel electrophoresis of PCR products and staining with ethidium bromide.

all tested pools, but *C. punctatus* could be identified in all investigated pools.

Conclusions

Our study yielded no evidence that *C. imicola* midges occurred in the study area in Germany. Members of the *C. obsoletus* group were detected in the entire monitoring area in high abundances and frequently contained BTV genome. Because of the detection of BTV in a high number of batches, which consisted of *C. obsoletus* sensu stricto, this species must be assumed to play a major role as a vector of BTV in Germany.

Acknowledgments

We thank Stefanie Bartsch, René Focke, Thomas Hörbrand, Sven Klimpel, Miriam Lang, Kathrin Lehmann, Christian Karl Meiser, Anja Stephan, Esther Timmermann, Bettina Vorsprach, Volker Walldorf, and Denis Wolf for their contributions. We also thank Petra Kranz for technical assistance in managing the midge database; Christoph Staubach for scientific advice; and Karin Lissek, Christian Korthase, and Katja Wittig for excellent technical assistance in the German National Reference Laboratory for Bluetongue Disease.

This study was supported by the German Federal Ministry of Food, Agriculture and Consumer Protection.

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References

1. Toussaint JF, Vandenbussche F, Mast J, De Meester L, Goris N, Van Dessel W, et al. Bluetongue in northern Europe. Vet Rec. 2006;159:327.
2. Elbers A, Backx A, van der Spek A, Ekker M, Leijs P, Steijn K, et al. Epidemiology of bluetongue virus serotype 8 outbreaks in the Netherlands in 2006. Tijdschr Diergeneeskd. 2008;133:222–9.
3. Toussaint JF, Sailleau C, Mast J, Houdart P, Czaplicki G, Demeestere L, et al. Bluetongue in Belgium, 2006. Emerg Infect Dis. 2007;13:614–6.
4. Saegerman C, Berkvens D, Mellor PS. Bluetongue epidemiology in the European Union. Emerg Infect Dis. 2008;14:539–44. DOI: 10.3201/eid1404.071441
5. Conraths FJ, Gethmann JM, Staubach C, Mettenleiter TC, Beer M, Hoffmann B. Epidemiology of bluetongue virus serotype 8, Germany. Emerg Infect Dis. 2009;15:433–5. DOI: 10.3201/eid1503.081210
6. Hoffmann B, Sasserath M, Thalheim S, Bunzenthal C, Strebelow G, Beer M. Bluetongue virus serotype 8 reemergence in Germany, 2007 and 2008. Emerg Infect Dis. 2008;14:1421–3. DOI: 10.3201/eid1409.080417
7. Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PPC, Baylis M. Climate change and the recent emergence of bluetongue in Europe. Nat Rev Microbiol. 2005;3:171–81. DOI: 10.1038/nrmicro1090
8. Meiswinkel R, Baldet T, de Deken R, Takken W, Delécolle JC, Mellor PS. The 2006 outbreak of bluetongue in northern Europe—the entomological perspective. Prev Vet Med. 2008;87:55–63. DOI: 10.1016/j.prevetmed.2008.06.005
9. Delecolle J-C. Nouvelle contribution à l'étude systématique et iconographique des espèces du genre *Culicoides* (Diptera:Ceratopogonidae) du Nord-Est de la France. Strasbourg (France): Université Louis Pasteur de Strasbourg; 1985.
10. Glukhova VM. Blood-sucking midges of the genera *Culicoides* and *Forcipomyia* (Ceratopogonidae) [in Russian]. Leningrad (Russia): Nauka; 1989.
11. Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. J Virol Methods. 2007;140:115–23. DOI: 10.1016/j.jviromet.2006.11.007
12. Nolan DV, Carpenter S, Barber J, Mellor PS, Dallas JF, Mordue Luntz AJ, et al. Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. Vet Microbiol. 2007;124:82–94. DOI: 10.1016/j.vetmic.2007.03.019
13. Mehlhorn H, Walldorf V, Klimpel S, Jahn B, Jaeger F, Eschweiler J, et al. First occurrence of *Culicoides obsoletus*–transmitted bluetongue virus epidemic in central Europe. Parasitol Res. 2007;101:219–28 [erratum in Parasitol Res 2007,101:833–4]. DOI: 10.1007/s00436-007-0519-6
14. Meiswinkel R, van Rijn P, Leijs P, Goffredo M. Potential new *Culicoides* vector in northern Europe. Vet Rec. 2007;161:564–5.

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Coxsackievirus A6 and Hand, Foot, and Mouth Disease, Finland

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During fall 2008, an outbreak of hand, foot, and mouth disease (HFMD) with onychomadesis (nail shedding) as a common feature occurred in Finland. We identified an unusual enterovirus type, coxsackievirus A6 (CVA6), as the causative agent. CVA6 infections may be emerging as a new and major cause of epidemic HFMD.

Hand, foot, and mouth disease (HFMD) is a common childhood illness characterized by fever and vesicular eruptions on hands and feet and in the mouth (Figure 1). It is caused by members of the family *Picornaviridae* in the genus *Enterovirus*. Complications are rare, but pneumonia, meningitis, or encephalitis may occur. Outbreaks of HFMD have been mainly caused by 2 types of enterovirus A species, coxsackievirus (CV) A16 (CVA16) or enterovirus 71 (*1*). Some outbreaks have been associated with CVA10, but only sporadic cases involving other members of the enterovirus A species have been reported (*2*,*3*).

During fall 2008, a nationwide outbreak of HFMD occurred in daycare centers and schools in Finland, starting in August and continuing at least until the end of the year and possibly into the following year. From vesicle fluid specimens of hospitalized children, we identified the etiologic agent as coxsackievirus A6.

The Study

In August 2008, vesicle fluid specimens were collected from 2 children and 1 parent with HFMD at the Central Hospital of Seinäjoki, Southern Ostrobothnia. Specimens were sent to the Department of Virology, University of Turku, for identification of the causative agent. After detection of CVA6 in these index cases, the virus was also found in specimens obtained from the Pirkanmaa Hospital District (Tampere), Turku University Hospital (Turku), Pori Central Hospital (Pori), and Central-Ostrobothnia Central Hospital (Kokkola) (Table).

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DOI: 10.3201/eid1509.090438

Nucleic acids were extracted from specimens by using the NucliSens EasyMag automated extractor (bioMèrieux, Boxtel, the Netherlands). When the extracts were analyzed for enteroviruses by using real-time reverse transcriptase–PCR (RT-PCR) specific for the 5′ noncoding region (NCR) of picornaviruses (*4*), amplicons with melting points indistinguishable from each other and typical to enteroviruses were obtained.

To identify the enterovirus type in the specimens, RT-PCR, specific for a partial sequence of the viral protein 1 (VP1) region, was performed by using the COnsensus-DEgenerate Hybrid Oligonucleotide (CODEHOP) Primers (bioinformatics.weizmann.ac.il/blocks/codehop.html) (*5*). The amplicons were separated by agarose gel electrophoresis, purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and sequenced in the DNA Sequencing Service Laboratory of the Turku Centre for Biotechnology. The virus type in the 3 index specimens, 3 samples of vesicular fluids, and 1 throat swab was successfully identified with sequencing and BLAST (www.ncbi.nlm.gov/BLAST) analysis as CVA6. Phylogenetic relationships of the sequences were examined by using CVA6 (Gdula strain), CVA16 (G10), and enterovirus 71 (BrCr) prototype strains as well as selected clinical CVA6 isolates obtained from GenBank. Sequence alignments were generated with the ClustalW program (www.ebi.ac.uk/clustalw), and the phylogenetic tree was computed by using the Jukes-Cantor algorithm and the neighbor-joining method. Phylogenetic analyses were conducted by using MEGA4 software (www.megasoftware.net) and the bootstrap consensus tree inferred from 1,000 replicates (*6*) (Figure 2).

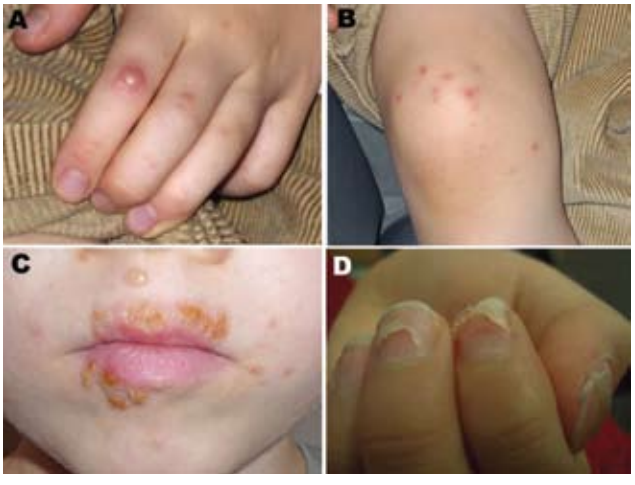

Figure 1. Vesicular eruptions in A) hand, B) foot, and C) mouth of a 6.5-year-old boy from Turku, Finland, with coxsackievirus (CV) A6 infection. Several of his fingernails shed 2 months after the pictures were taken. D) Onychomadesis in a 10-year-old boy from Seinäjoki, Finland, 2 months after hand, foot and mouth disease with CVA6 infection. Photographs courtesy of H. Kujari (A–C) and M. Linna (D).

Table. Laboratory findings in clinical specimens and epidemiologic data for patients with CVA6 infections, Finland, 2008*

City or place, identification	Sampling date	Sex/age, y	Specimen type	Disease or signs	CVA6-VP1 RT-PCR	VP1 sequence	5′ NCR sequence
Seinäjoki							
Fin/Se8717	2008 Aug	M/1.3	Vesicle fluid	HFMD	Pos	CVA6	CVA6
Fin/Se8781	2008 Aug	F/34	Vesicle fluid	HFMD	Pos	CVA6	CVA6
Fin/Se8841	2008 Aug	M/0.9	Vesicle fluid	HFMD	Pos	CVA6	CVA6
Fin/Se8865	2008 Aug	†	Feces	HFMD	Pos	NR	CVA6
Fin/Se8913	2008 Sep	M/10	Throat swab	HFMD	Pos	NR	CVA6
Fin/Se8925	2008 Sep	M/0.9	Throat swab	HFMD	Pos	NR	CVA6
Fin/Se8926	2008 Sep	†	Vesicle fluid	HFMD	Pos	CVA6	CVA6
Fin/Se8927	2008 Sep	F/1.3	Throat swab	HFMD	Pos	NR	CVA6
Fin/Se8928	2008 Sep	†	Vesicle fluid	HFMD	Pos	CVA6	CVA6
Fin/Se8931	2008 Sep	M/0.8	Throat swab	NA	Pos	NR	CVA6
Turku							
Fin/Tu8859	2008 Sep	M/5.8	Throat swab	Fever, tonsillitis	Pos	CVA6	CVA6
Fin/Tu8866	2008 Sep	M/3.1	Throat swab	HFMD	Pos	NR	CVA6
Fin/Tu81027	2008 Oct	M/1.8	Throat swab	HFMD	Pos	NR	CVA6
Fin/Tu81042	2008 Oct	M/1.8	Throat swab	Fever, eczema	Pos	NR	CVA6
Fin/Tu81038	2008 Oct	F/2.2	Throat swab	Fever, seizure	Pos	NR	CVA6
Fin/Tu81274	2008 Nov	M/6.5	Vesicle fluid	HFMD	Pos	ND	CVA6
Fin/Tu81309	2008 Dec	F/3.7	Vesicle fluid	HFMD	Pos	ND	CVA6
Fin/Tu81321	2008 Dec	F/1.2	Throat swab	HFMD	Pos	ND	NR
Fin/Tu963	2009 Jan	M/1.2	Throat swab	HFMD	Pos	ND	CVA6
Fin/Tu/IB	2009 Feb	F/5.7	Nail	Recent HFMD	Pos	ND	CVA6
Pori							
Fin/Po8959	2008 Oct	M/4.8	Throat	Fever, vomiting	Neg	ND	CVA6
Fin/Po81375	2008 Dec	M/0.5	Vesicle fluid	HFMD	Pos	ND	CVA6
Fin/Po81376	2008 Dec	†	Throat	HFMD	Pos	ND	CVA6
Fin/Po81324	2008 Dec	M/10	Feces	Fever, eczema	Pos	ND	NR
Fin/Po81325	2008 Dec	M/10	Feces	Fever, HFMD contact	Pos	ND	NR
Tampere							
Fin/Ta8966	2008 Sep	M/3.1	Tracheal aspirate	NA	Pos	NR	CVA6
Fin/Ta81145	2008 Sep	M/2.1	Feces	NA	Pos	NR	NR
Fin/Ta81074	2008 Oct	M/0.3	Tracheal aspirate	NA	Pos	ND	CVA6
Fin/Ta81125	2008 Oct	F/0.7	Vesicle fluid	HFMD	Pos	CVA6	CVA6
Fin/Ta81126	2008 Oct	M/0.3	Vesicle fluid	HFMD	Pos	NR	CVA6
Fin/Ta81216	2008 Nov	F/2.0	Throat swab	NA	Pos	ND	CVA6
Fin/Ta81252	2008 Nov	M/6.1	Throat swab	NA	Pos	ND	CVA6
Kokkola							
Fin/Ko937	2009 Jan	M/10	Vesicle fluid	HFMD	Pos	ND	CVA6

*CVA6, coxsackievirus A6; VP1, virus protein 1; RT-PCR, reverse transcriptase–PCR; NCR, noncoding region; HFMD, hand, foot, and mouth disease; pos, positive; neg, negative; NA, not available; ND, not done; NR, no result (VP1 sequencing was attempted without result).
†Same patient as on the line above.

Phylogenetic analysis placed all CVA6 strains from the HFMD outbreak in 1 cluster (97%–100% identity), whereas the nucleotide identities between those isolates and CVA6 prototype strains Gdula, CAV16, G-10, and enterovirus 71 BrCr were 82.5%–83.2%, 55.6%–56.6%, and 55.6%–57.3%, respectively. The closest preceding CVA6 strain was isolated from cerebrospinal fluid in the United Kingdom in 2007 and had 92%–94% nucleotide identity with the strains described here (*7*).

To improve the detection of the novel CVA6 strains in clinical specimens, we designed specific VP1 primers from the aligned sequences. CVA6vp1 reverse primer (5′-ACTCGCTGTGTGATGAATCG-3′) and CVA6vp1 forward primer (5′-CGTCAAAGCGCATGTATGTT-3′) generated a 199-bp amplicon. First, cDNA was synthesized in a 20-μL reaction mixture containing 1 μmol/L CVA6vp1 reverse primer, 2.5 mmol/L of each dNTP, 20 U RevertAid H Minus M-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany), reaction buffer (Fermentas), 4 U RiboLock RNase inhibitor (Fermentas), and 5 μL RNA incubated at 42ºC for 1 h. Then, 5 μL of cDNA was added to 20 μL of master mixture containing 0.4 μmol/L each of the CVA6vp1 primers and Maxima SYBR Green qPCR Master Mix (Fermentas). PCR with melting curve analysis was performed in a Rotor-Gene 6000 real-time instrument (Corbett Research, Mortlake, Victoria, Australia) by

Figure 2. Phylogenetic analysis of coxsackievirus (CV) A6 partial (289 bp) viral protein 1 sequences showing the relationships between the recent clinical CVA6 samples isolated in Finland (triangles), selected CVA6 isolates from GenBank, and prototypes of CVA6, CVA16, and enterovirus (EV) 71. GenBank accession numbers are included. Scale bar indicates nucleotide substitutions per position.

using the following cycling conditions: initial denaturation at 95ºC for 10 min, 45 cycles at 95ºC for 15 s, 60ºC for 30 s, and at 72ºC for 45 s, followed by generation of melting curve from 72°C to 95°C with temperature increments of 0.5°C/s. Partial 5′ NCR sequence of the strains in clinical specimens was determined as described (*4*) and compared with the known sequences by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

During autumn 2008, a total of 47 acute-phase specimens, including 12 vesicle fluid samples, 23 throat swabs, 2 tracheal aspirates, 5 fecal samples, and 5 cerebrospinal fluid specimens from 43 patients yielded amplicons with similar melting points as the originally identified CVA6 strains in 5′ NCR RT-PCR. All specimens were subjected to the specific CVA6-VP1 real-time RT-PCR, and a positive result was obtained for 11 vesicle fluid samples, 14 throat swabs, 2 tracheal aspirates, and 4 fecal samples (Table). The virus in 1 throat swab was identified as CVA6 from the result of 5′ NCR sequencing alone. None of the CVA6-positive specimens were positive by an RT-PCR assay with CVA16- and EV71-specific primers (*8*). Attempts to cultivate the virus from 8 CVA6 RT-PCR-positive specimens were unsuccessful, whereas the prototype strain could be propagated in rhabdomyosarcoma cells.

Onychomadesis was 1 characteristic feature in patients during this HFMD outbreak; parents and clinicians reported that their children shed fingernails and/or toenails within 1–2 months after HFMD (Figure 1). Only a few published reports of nail matrix arrest in children with a clinical history of HFMD exist in the medical literature (*9*–*11*). We obtained shed nails from 2 siblings who had HFMD 8 weeks before the nail shedding. The nail fragments were stored at –70°C for a few weeks and treated with proteinase K before nucleic acid extraction. The extracts were enterovirus positive in 5′ NCR RT-PCR. The virus in one of them was identified as CVA6 by the specific RT-PCR and yielded a 5′ NCR sequence that was similar to the novel CVA6 strains.

Conclusions

Enterovirus CVA6 was a primary pathogen associated with HFMD during a nationwide outbreak in Finland in autumn of 2008. HFMD epidemics have primarily been associated with CVA16 or enterovirus 71 infections; those caused by enterovirus 71 have occurred more frequently in Southeast Asia and Australia in recent years (*12*). Reportedly, CVA10 has been found in minor outbreaks; other coxsackievirus A types have been found in only sporadic cases of HFMD (*2,3*). In general, CVA6 infections have been seldom detected and mostly in association with herpangina (*13,14*). In Finland, CVA6 has been identified only on 4 occasions over 8 years during enterovirus surveillance from 2000 to 2007 (*15*).

Although the CODEHOP primers were elementary for rapid genotyping of the novel CVA6 strains, we identified more viruses with the designated CVA6-VP1 specific primers. Onychomadesis was a hallmark of this HFMD outbreak. To our surprise, we detected CVA6 also in a fragment of shed nail. The same virus could have given rise to the outbreak in Spain in 2008 (*10*). Supposedly, virus replication damages nail matrix and results in temporary nail dystrophy. Whether nail matrix arrest is specific to CVA6 infections remains to be shown. This study demonstrates that CVA6, in addition to CVA16 and enterovirus 71, may be emerging as a primary cause of HFMD.

Acknowledgments

We thank Heidi Berghäll for nail samples, Harry Kujari for photographs, and Tiina Ylinen for assistance in the laboratory.

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References

1. McMinn PC. An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol Rev. 2002;26:91–107. DOI: 10.1111/j.1574-6976.2002.tb00601.x

2. Kamahora T, Itagaki A, Hattori N, Tsuchie H, Kurimura T. Oligonucleotide fingerprint analysis of coxsackievirus A10 isolated in Japan. J Gen Virol. 1985;66:2627–34. DOI: 10.1099/0022-1317-66-12-2627
3. Cabral LA, Almeida JD, de Oliveira ML, Meza AC. Hand, foot, and mouth disease case report. Quintessence Int. 1998;29:194–6.
4. Peltola V, Waris M, Österback R, Susi P, Ruuskanen O, Hyypiä T. Rhinovirus transmission within children: incidence of symptomatic and asymptomatic infections. J Infect Dis. 2008;197:382–9. DOI: 10.1086/525542
5. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol. 2006;44:2698–704. DOI: 10.1128/JCM.00542-06
6. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, editor. Mammalian protein metabolism. New York: Academic Press; 1969. p. 21–132.
7. Leitch EC, Harvala H, Robertson I, Ubillos I, Templeton K, Simmonds P. Direct identification of human enterovirus serotypes in cerebrospinal fluid by amplification and sequencing of the VP1 region. J Clin Virol. 2009;44:119–24. DOI: 10.1016/j.jcv.2008.11.015
8. Xiao XL, He YQ, Yu YG, Yang H, Chen G, Li HF, et al. Simultaneous detection of human enterovirus 71 and coxsackievirus A16 in clinical specimens by multiplex real-time PCR with an internal amplification control. Arch Virol. 2009;154:121–5. DOI: 10.1007/s00705-008-0266-8
9. Bernier V, Labrèze C, Bury F, Taïeb A. Nail matrix arrest in the course of hand, foot and mouth diease. Eur J Pediatr. 2001;160:649–51.
10. Clementz GC, Mancini AJ. Nail matrix arrest following hand-foot-mouth disease: a report of five children. Pediatr Dermatol. 2000;17:7–11. DOI: 10.1046/j.1525-1470.2000.01702.x
11. Salazar A, Febrer I, Guiral S, Gobernado M, Pujol C, Roig J. Onychomadesis outbreak in Valencia, Spain, June 2008. Euro Surveill. 2008;13:pii:18917. Available from http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18917
12. McMinn P, Lindsay K, Perera D, Chan HM, Chan KP, Cardosa MJ. Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. J Virol. 2001;75:7732–8. DOI: 10.1128/JVI.75.16.7732-7738.2001
13. Grist NR, Bell EJ, Assaad F. Enteroviruses in human disease. Prog Med Virol. 1978;24:114–57.
14. Yamashita T, Ito M, Taniguchi A, Sakae K. Prevalence of coxsackievirus A5, A6, and A10 in patients with herpangina in Aichi Prefecture, 2005. Jpn J Infect Dis. 2005;58:390–1.
15. Blomqvist S, Paananen A, Savolainen-Kopra C, Hovi T, Roivainen M. Eight years of experience with molecular identification of human enteroviruses. J Clin Microbiol. 2008;46:2410–3. DOI: 10.1128/JCM.00313-08

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Hantavirus Infection in the Republic of Georgia

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We describe a laboratory-confirmed case of hantavirus infection in the Republic of Georgia. Limited information is available about hantavirus infections in the Caucasus, although the infection has been reported throughout Europe and Russia. Increasing awareness and active disease surveillance contribute to our improved understanding of the geographic range of this pathogen.

Rodent-borne hantavirus infections causing hemorrhagic fever with renal syndrome (HFRS) occur throughout most of Europe and Russia. The pathogenic hantaviruses detected in Europe and Russia include Puumala, Dobrava, Saaremaa, Tula, Amur, and Hantaan viruses (*1,2*). Seroprevalence studies of hantavirus infection in Europe have shown prevalence rates as high as 6%–9% in Sweden, Estonia, and European Russia (*1*). The circulation of hantaviruses is specific predominantly to rodent species and thus correlates with the host range of the rodent reservoir. Several lineages of Dobrava virus circulate in Europe and Russia (*3*–*6*), and are named according to their rodent reservoirs: *Apodemus flavicollis* (Dobrava-Af), *A. agrarius* (Dobrava-Aa), and *A. ponticus* (Dobrava-Ap). Dobrava-Aa is suspected to be the predominant strain in central Europe (*7*).

The clinical manifestations of hantavirus infection vary and depend largely on the strain of the infecting virus. Classic HFRS is characterized by fever, acute renal failure, hypotension, hemorrhage, and vascular leakage. Puumala virus typically induces a mild variant of HFRS (nephropathia epidemica) accompanied by high fever, headache, backache, and abdominal pain. Mild hemorrhagic manifestations, including conjunctival hemorrhage or petechiae on the trunk or palate, also may occur. Severe clinical manifestations are rare (<1% of patients) but can include acute renal failure, severe neurologic manifestations, and death (*8*). Dobrava-Aa may induce a similarly mild clinical course of illness to that of Puumala virus (*6*).

Information is scant about hantaviruses in the Republic of Georgia. Although reports of hantavirus infection from other countries in the surrounding area suggest that this pathogen circulates in the Caucasus, the incidence of these infections is unknown.

The Patient

A 31-year-old male resident of Tbilisi, Georgia, sought treatment at the Infectious Pathology, AIDS and Clinical Immunology Center in early June 2008, six days after onset of illness. At that time, he was enrolled in an ongoing laboratory-based surveillance study of acute febrile illness (AFI). Initial clinical symptoms were fever (maximum axillary temperature 39°C), arthralgias and myalgias in the lower extremities, back pain, vomiting, and diffuse abdominal pain. Vital signs indicated a pulse rate of 87 bpm and blood pressure of 140/80 mm Hg. The physical examination noted mild hepatosplenomegaly and pharyngeal injection. The chest radiograph and ultrasound of the abdomen were normal. No preexisting illnesses were reported. Travel history included regular trips to Marneuli district (a rural district south of Tbilisi). No specific rodent exposures were noted, and no other travel was reported.

Laboratory findings were normal, with the exception of elevated band neutrophils (20%, reference range 1%–6%) and lymphocytosis (44%, reference range 19%–37%). C-reactive protein level was elevated (48.9 mg/mL, reference range <6 mg/mL), and decreased total protein (62 g/L, reference range 65–85 g/L) and albumin levels (20 g/L, reference range 35–50 g/L) were observed. Results of liver function tests and serum creatinine levels were within normal limits.

An elevated creatinine level (214 μmol/L, reference range 53–115 μmol/L) was noted on day 9 of illness, along with a decrease in urine output. Proteinuria (3 g/24 h), microscopic hematuria, and elevated blood pressure (160/90 mm Hg) were also observed. The patient was transferred to the nephrology department of the Institute of Urology in Tbilisi with a diagnosis of acute renal failure. Patient's fluid input and output were closely regulated, and his condition improved gradually without dialysis.

Serum samples were tested for antibodies against a panel of pathogens, including *Salmonella enterica* serovar Typhi, *S. paratyphi* A and B, Epstein-Barr virus, *Brucella* spp., *Coxiella burnetti*, hantavirus, *Rickettsia* (spotted fever and scrub typhus groups), West Nile virus, tick-borne encephalitis virus, and *Leptospira* spp. All serologic test results were negative except for the test result for hantavirus. To corroborate hospital laboratory diagnostics, serum

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DOI: 10.3201/eid1509.090617

samples were also tested in a private laboratory in Germany by using Western blot assay (recomBlot Bunyavirus immunoglobulin [Ig]G/IgM; Mikrogen, Neuried, Germany) and immunofluorescence antibody (IFA) assay (Progen, Heidelberg, Germany). Results from the Western blot assay were negative for Puumala IgG and positive for Hantaan IgG and Dobrava IgM. The IFA result for Hantaan antibodies was positive at a reciprocal titer >2,048. The Naval Medical Research Unit No. 3 in Egypt conducted a hantavirus IgM ELISA (Focus Diagnostics, Cypress, CA, USA) on paired serum samples (acute-phase sample positive:negative ratio = 8.42; convalescent phase = 7.815, cut-off 1.10). Results of blood and *Leptospira* spp. cultures were negative. A renal biospy specimen obtained 10 days after disease onset showed acute tubular necrosis with mild-grade arteriolosclerosis (Figure).

On the 11th day of illness, the patient's general condition started to improve, with increased urine output, resolution of abdominal pain, and normalization of blood pressure (120/70 mm Hg). No sequelae were reported at 1-month follow-up.

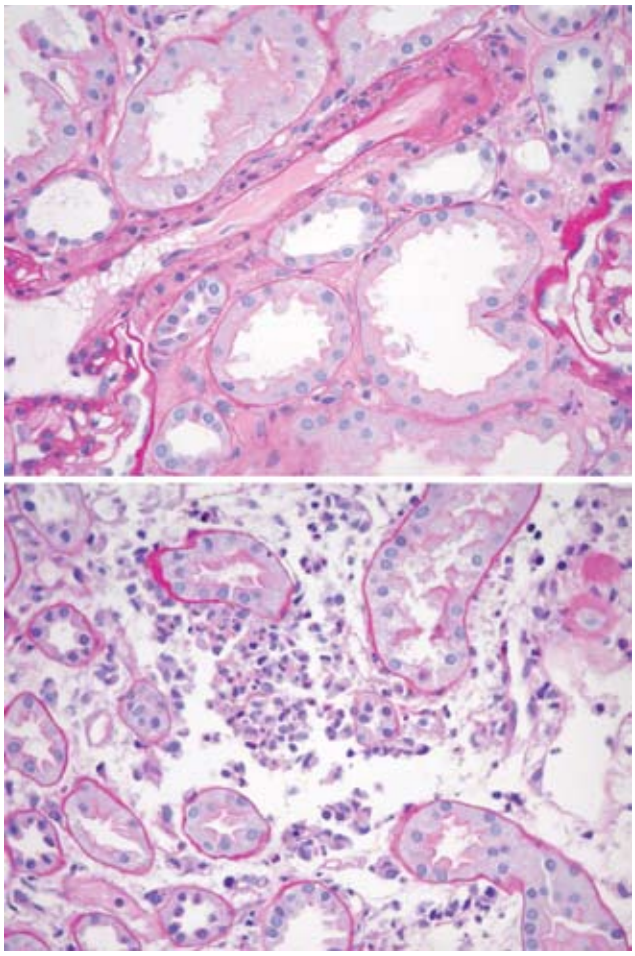

Figure. Acute tubular necrosis in a renal biopsy specimen of the patient. Magnification ×40.

Conclusions

The clinical presentation and results of serologic tests for this patient who had renal failure but no apparent hemorrhage were consistent with results previously described for hantavirus infection. Although the differential diagnosis included other infectious and noninfectious causes of renal failure including leptospirosis, ongoing surveillance of AFI in Georgia provided the serologic testing capability that enabled clinicians to identify a hantavirus infection as the cause of this illness characterized by high fever and evidence of renal dysfunction (increased serum creatinine, proteinuria, and hematuria).

The pathologic changes observed in hantavirus infection presumably result from increased vascular permeability, characterized clinically by elevated hematocrit and decreased serum protein levels. Damage to the vascular endothelium along with cytokine-induced renal tubular and interstitial pathologic changes may partially explain renal failure associated with hantavirus infection. Although the most commonly noted renal pathologic finding is acute interstitial nephritis, acute tubular cell necrosis is also described. A comparative study of Puumala and Dobrava renal pathology noted increased intensity and extent of medullary interstitial capillary injury, hemorrhages, and tubular necrosis among Dobrava virus-infected patients (*9*).

The strain of the infecting virus is unknown, but serologic results, along with the available information from surrounding countries, implicate Dobrava virus as the infecting strain. The relatively mild clinical symptoms of this patient suggest that a variant of Dobrava virus, or a closely related hantavirus strain that cross-reacts with Dobrava, may be the etiologic agent. Information about the circulating virus strains, clinical manifestation, rodent hosts, and disease prevalence are lacking for the Caucasus. Further studies are needed on potential rodent hosts and characterization of circulating strains.

Acknowledgments

We thank Marina Endeladze, Irma Tchokhonelidze, and all participants of the Acute Febrile Illness Surveillance Network in Georgia for their contributions to this study.

This study was supported by the US Department of Defense, Global Emergence Infections Surveillance Program, and by the Centers for Disease Control and Prevention, International Emerging Infections Program in Egypt.

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References

1. Vapalahti O, Mustonen J, Lundkvist E, Henttonen H, Plyusnin A, Vaheri A. Hantavirus infections in Europe. Lancet Infect Dis. 2003;3:653–61. DOI: 10.1016/S1473-3099(03)00774-6
2. Heyman P, Vaheri A, the European Network for diagnostics of Imported Viral Diseases (ENIVD). Situation of hantavirus infections and haemorrhagic fever with renal syndrome in European countries as of December 2006. Euro Surveill. 2008;13:pii 18925.
3. Klempa B, Schutt M, Auste B, Labuda M, Ulrich R, Meisel H, et al. First molecular identification of human Dobrava virus infection in Central Europe. J Clin Microbiol. 2004; 42:1322–5. DOI: 10.1128/JCM.42.3.1322-1325.2004
4. Nemirov K, Vapalahti O, Lundkvist A, Vasilenko V, Golovljova I, Plyusnina A, et al. Isolation and characterization of Dobrava hantavirus carried by the striped field mouse (*Apodemus agrarius*) in Estonia. J Gen Virol. 1999;80:371–9.
5. Klempa B, Schmidt HA, Ulrich R, Kaluz S, Labuda M, Meisel H, et al. Genetic interaction between distinct Dobrava hantavirus subtypes in *Apodemus agrarius* and *A. flavicollis* in nature. J Virol. 2003; 77:804–9. DOI: 10.1128/JVI.77.1.804-809.2003
6. Klempa B, Tkachenko EA, Dzagurova TK, Yunicheva YV, Morozov VG, Okulova NM, et al. Hemorrhagic fever with renal syndrome caused by 2 lineages of Dobrava hantavirus, Russia. Emerg Infect Dis. 2008;14:617–25.
7. Klempa B, Stanko M, Labuda M, Ulrich R, Meisel H, Kruger DH. Central European Dobrava hantavirus isolate from a striped field mouse (*Apodemus agrarius*). J Clin Microbiol. 2005; 43:2756–63. DOI: 10.1128/JCM.43.6.2756-2763.2005
8. Muranyi W, Bahr U, Zeier M, van der Woude FJ. Hantavirus infection. J Am Soc Nephrol. 2005;16:3669–79. DOI: 10.1681/ASN.2005050561
9. Ferluga D, Vizjak A. Hantavirus nephropathy. J Am Soc Nephrol. 2008;19:1653–8. DOI: 10.1681/ASN.2007091022

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Highly Pathogenic Avian Influenza Virus A (H7N3) in Domestic Poultry, Saskatchewan, Canada, 2007

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Epidemiologic, serologic, and molecular phylogenetic methods were used to investigate an outbreak of highly pathogenic avian influenza on a broiler breeding farm in Saskatchewan, Canada. Results, coupled with data from influenza A virus surveillance of migratory waterfowl in Canada, implicated wild birds as the most probable source of the low pathogenicity precursor virus.

Wild aquatic birds of the orders Anseriformes and Charadriiformes are the natural reservoir for influenza A viruses (*1*) and are thought to serve as a source of virus that leads to outbreaks in domestic poultry. However, direct evidence for this suggestion is often difficult to demonstrate.

On September 22, 2007, a broiler hatching egg operation near Regina Beach, Saskatchewan, Canada, experienced a sudden increase in deaths (140 [36%] of 390 birds) in a barn that housed 24-week-old roosters. The premises contained 53,000 birds of multiple ages housed in 10 confinement barns. On September 23, deaths in the rooster barn increased to 240 (62%). Postmortem examination findings, which showed lesions compatible with highly pathogenic avian influenza (HPAI), resulted in a Canadian Food Inspection Agency team being dispatched to the premises. The farm was placed under quarantine, and specimens were submitted to the regional Avian Influenza Network laboratory in Saskatoon, Saskatchewan, and the National Centre for Foreign Animal Disease in Winnipeg, Manitoba, for diagnosis. A complete account of the index premises, disease control actions, and description of the Saskatchewan poultry industry is online at www.inspection.gc.ca/english/anima/heasan/disemala/avflu/2007sask/repsaske.shtml.

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DOI: 10.3201/eid1509.080231

The Study

Six pools (5 samples per pool) of cloacal swab specimens, 6 pools (5 samples per pool) of oropharyngeal swab specimens, 6 pools of 10% (wt/vol) tissue (heart, liver, lung, and spleen), 6 pools of intestine homogenates, and 2 pools of brain homogenates were tested by using real-time reverse transcription–PCR (RT-PCR) assays specific for the influenza A virus matrix gene (*2*), H5 and H7 hemagglutinin (HA) subtype genes (*2*), and avian paramyxovirus serotype-1 matrix gene (*3*). Virus isolation was performed by using embryonating chicken eggs according to international standards (*4*). RT-PCR of swab and tissue samples showed positive results for influenza A matrix but negative results for H5 and H7 subtypes and avian paramyxovirus serotype-1.

Because of apparent inconsistencies between these initial results and clinical signs observed on the farm, further analyses were conducted by using conventional RT-PCR assays with universal primers designed to amplify the complete HA gene and the 9 neuraminidase gene subtypes of avian influenza virus (*5*). Results from these ancillary tests showed evidence for an avian influenza virus (H7N3), which was subsequently confirmed by virus isolation and subtyping by hemagglutination-inhibition and neuraminidase-inhibition assays (*4*).

Viral isolates grew well in the chicken host, producing HA titers as high as 1,024. The derived amino acid sequence of the HA_0 cleavage site, PENPKTTKPRPRR/GLF, (underlined amino acids indicate a 6-aa insert) conformed to the definition of the World Organisation for Animal Health for HPAI virus (*4*). Intravenous inoculation of 4- to 6-week-old chickens (*4*) with isolate A/chicken/Saskatchewan/HR-00011/2007 resulted in all birds dying within 24 hours, giving an intravenous pathogenicity index of 3.0. This finding confirmed the molecular pathotype. Tissues from dead roosters showed specific influenza A virus immunolabeling in all organs examined, including the central nervous system, a characteristic of HPAI.

Negative real-time RT-PCR results for H7 were explained by the presence of 8-nt substitutions within the primer and probe target sites: 2 and 1 in the forward and reverse primers, respectively, and 5 in the probe. This real-time RT-PCR assay for H7 (*2*) did not detect several H7 viruses subsequently isolated from wild birds in 2007, a finding that has also been reported Xing et al. (*6*).

Epidemiologic investigations conducted on poultry farms surrounding the index premises, including 6 farms located within the 3-km surveillance zone, showed no evidence of avian influenza virus infection. Sharing of equipment, movement of employees among the barns, and lack of designated footwear or clothing for each employee on the index farm increased the likelihood of inadvertent introduction of environmental contaminants. The barns used a municipal water source, but during high demand periods surface water from a dugout located ≈380 m from the breeder barns was also used. This water was routinely filtered and treated with ozone, but a failure of the ozonater was reported during July. Several small natural water bodies are also located near the premises, the closest being 1,100 m away. Last Mountain Lake, which has a length of 80 km and a waterfowl staging area at its northern end, is located 5.5 km away.

Serologic testing was conducted to evaluate the length of time an avian influenza virus (H7N3) had been circulating on the premises. Of serum samples obtained from 24-week-old roosters on September 23, 62% (18/29) had antibodies against avian influenza virus nucleoprotein (NP) (*7*); all were negative for antibodies against H7 (*4*). Analysis of serum samples from surviving roosters 3 days later showed that 90% (18/20) had antibodies against NP and 84% (16/19) had antibodies against H7. In contrast, 100% (21/21) of 32-week-old breeder hens had antibodies against NP and 93% (14/15) had antibodies against H7, and 95% (19/20) of 55-week-old breeder hens had antibodies against NP and 87% (14/16) had antibodies against H7. These results suggest that breeders had been infected longer than roosters.

Serum samples that had been obtained from the 55-week-old flock on May 11, 2007, and the 32-week-old flock on June 8, 2007, were negative for antibodies against NP, which indicated that virus introduction occurred subsequently. Although samples from 55-week-old breeders had high H7 antibody titers, none of these birds showed overt clinical signs, which implied exposure to an avian influenza virus (H7N3) with low pathogenicity. Of note, ≈25% of 32-week-old breeders were clinically ill on September 28 (5 days after the initiation of quarantine), despite having some of the highest H7-specific antibody titers (Figure 1).

Phylogenetic analysis (Figure 2; Tables 1, 2) showed a close relationship of Saskatchewan/2007 H7N3 with recent North American H7 subtype viruses of free-flying waterfowl origin (*11*). Several of these viruses were isolated during an avian influenza surveillance program that had been coordinated since 2005 by the Canadian Cooperative Wildlife Health Centre. Although the wild bird surveillance program in Canada did not detect H7 viruses in 2005 (*12*) or 2006, a conclusion based on characterization

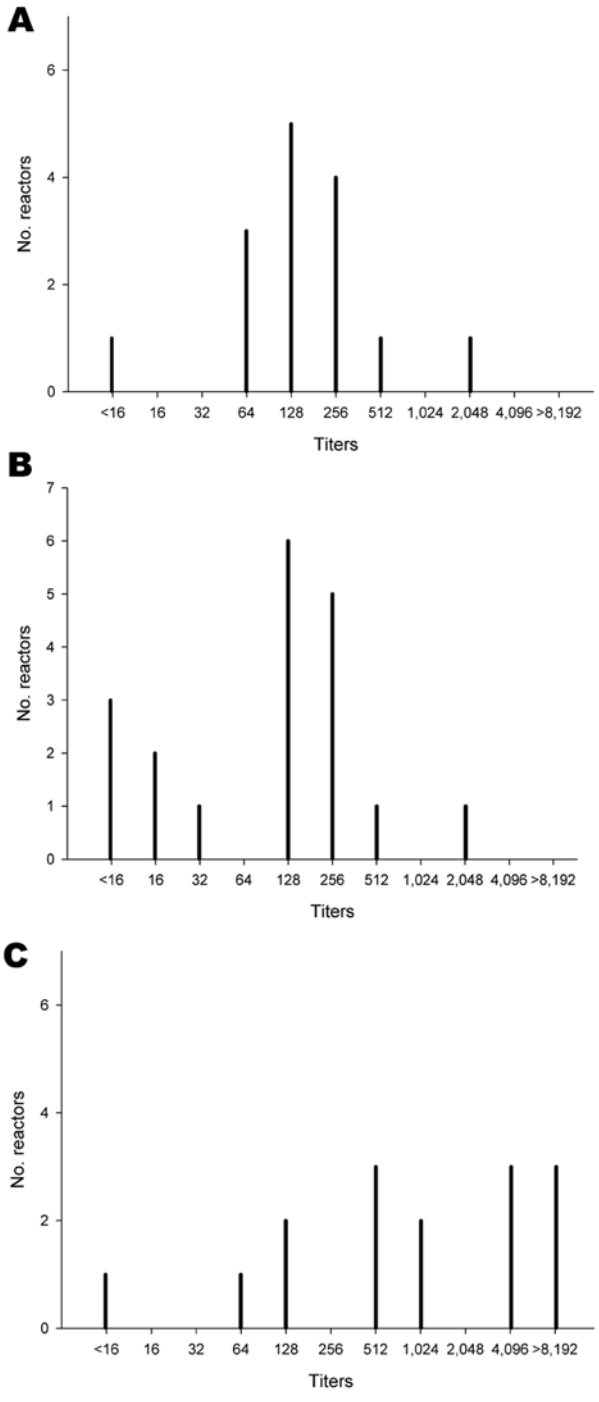

Figure 1. Avian influenza virus H7-specific antibody titers of serum samples from 55-week-old breeder chickens (A), 24-week-old roosters (B), and 32-week-old breeder chickens (C), Saskatchewan, Canada, September 26, 2007. Titers of individual birds were determined by the ability of 2-fold serial serum dilutions to inhibit agglutination 0.5% (vol/vol) chicken erythrocyte suspensions by 4 hemagglutination inhibition units of avian influenza virus (H7N3) A/chicken/British Columbia/2004.

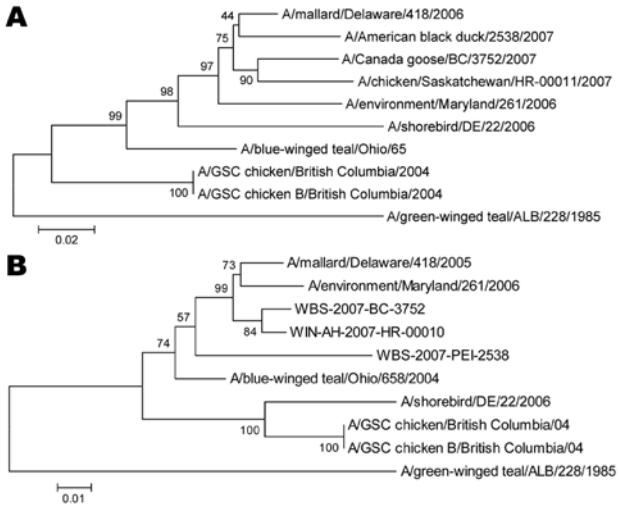

Figure 2. Phyogenetic analysis of avian influenza virus H7 (A) and N3 (B) genes. Trees were generated with MEGA software (*8*) by using the neighbor-joining method (*9*). Evolutionary distances were computed by using the method of Nei and Gojobori (*10*). Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Scale bars indicate substitutions per site.

of viruses that were isolated from all real-time RT-PCR swab samples positive for virus matrix gene, several H7 virus isolates were obtained in 2007. Most of these viruses were isolated from birds sampled in the neighboring provinces of British Columbia, Alberta, and Manitoba. The HA gene of Saskatchewan/2007 clusters with these 2007 wild bird isolates but not with the HA gene of A/chicken/British Columbia/2004 (H7N3), which was responsible for the HPAI outbreak in British Columbia. This finding further supports the hypothesis that the Saskatchewan/2007 isolate was of wild bird origin.

Conclusions

Potential breaches in biosecurity and proximity of the farm to a waterfowl habitat point to wild aquatic birds as the most likely virus source. Serologic evidence suggests that a low pathogenicity avian influenza virus (H7N3) circulated among breeder hens before roosters were exposed. Infection of 24-week-old roosters was likely associated with their movement into breeder barns on September 13, 18, and 19. This finding coincided with evolution of an HPAI virus by a process that may have involved nonhomologous recombination similar to that described for the HPAI (H7N3) outbreak in British Columbia (*13*). The ori-

Table 1. Comparison of 8 gene segments of avian influenza virus (H7N3) A/chicken/Saskatchewan/HR-00011/2007 with influenza virus genes from GenBank with highest sequence identity, Saskatchewan, Canada, 2007*

Gene	Nucleotide identity	%	Amino acid identity	%
PB2	A/laughing gull/Delaware/42/2006 (H7N3)	98	A/mallard/Ohio/656/2002 (mixed)	98
PB1	A/mallard/Alberta/149/2002 (H2N4)	98	A/mallard/Alberta/149/2002 (H2N4)	99
PA	A/snow goose/Maryland/353/2005 (H6N1)	98	A/mallard/Ohio/249/98 (H6N1)	99
H7	A/mallard/Delaware/418/2005 (H7N3)	96	A/environment/Maryland/566/2006 (H7N9)	97
NP	A/environment/Maryland/1176/2005 (H3N6)	98	A/GSC_chicken/British Columbia/2004 (H7N3)	99
N3	A/mallard/Delaware/418/2005 (H7N3)	98	A/environment/Maryland/261/2006 (H7N3)	98
M1	A/mallard/Maryland/1131/2005 (H12N5)	98	A/chicken/Korea/S6/2003 (H3N2)	98
M2	A/chicken/Singapore/93 (H10N5)	98	A/mallard duck/Alberta/376/85 (H2N3)	98
NS1	A/mallard/Alberta/30/2001 (H4N8)	98	A/pintail/Alaska/779/2005 (H3N8)	98
NS2	A/blue-winged teal/Louisanna/240B/88 (H4N6)	99	A/mallard/Delaware/418/2005 (H7N3)	98

*PB2, RNA polymerase subunit B2; PB1, RNA polymerase subunit B1; PA, RNA polymerase subunit A; H7, hemagglutinin 7; NP, nucleoprotein; N3, neuraminidase 3; M1, matrix protein 1; M2, matrix protein 2; NS1, nonstructural protein 1; NS2, nonstructural protein 2.

Table 2. Comparison of 8 gene segments of avian influenza virus (H7N3) A/chicken/Saskatchewan/HR-00011/2007 with 2 recent viruses (H7N3) isolated from wild waterfowl, Saskatchewan, Canada, 2007*

	A/American black duck/New Brunswick/2538/2007		A/Canada goose/British Columbia/3752/2007	
Gene	% nt identitiy	% aa identity	% nt identity	% aa identity
PB2	92	97.8	92	97.8
PB1	97	99.2	97	99.1
PA	98	95.5	97	95.4
H7	96	96.3	97	97
NP	94	97.4	94	97.2
N3	96	98.3	98	98.3
M1	96	98.8	97	98.8
M2	98	97.9	99	100
NS1	80	68.7	80	69.1
NS2	80	82.5	80	82.5

*GenBank accession nos. EU500841–EU500864. PB2, RNA polymerase subunit B2; PB1, RNA polymerase subunit B1; PA, RNA polymerase subunit A; H7, hemagglutinin 7; NP, nucleoprotein; N3, neuraminidase 3; M1, matrix protein 1; M2, matrix protein 2; NS1, nonstructural protein 1; NS2, nonstructural protein 2.

gin of the 6-aa insert within the HA_0 cleavage site remains speculative; a hypothetical protein of *Gallus gallus* (GenBank accession no. XM_424122) was 1 notable potential donor. The findings of this and other studies (*6*) emphasize the need for continually monitoring HA subtype-specific real-time RT-PCR assay performance, particularly when used in national avian influenza surveillance programs.

Acknowledgments

We thank Kevin Tierney, William Swiderski, Colleen Cottam-Birt, Marsha Leith, and Kevin Heather for excellent technical assistance.

R.G.W. was supported in part by the National Institute of Allergies and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract no. HHSN 266200700005C.

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References

1. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evoution and ecology of influenza A viruses. Microbiol Rev. 1992;56:152–79.
2. 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 avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 2002;40:3256–60. DOI: 10.1128/JCM.40.9.3256-3260.2002
3. Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, et al. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J Clin Microbiol. 2004;42:329–38. DOI: 10.1128/JCM.42.1.329-338.2004
4. World Organisation for Animal Health. Highly pathogenic avian influenza. In: Manual of diagnostic tests and vaccines for terrestrial animals. Paris: The Organisation; 2004. p. 258–69.
5. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol. 2001;146:2275–89. DOI: 10.1007/s007050170002
6. Xing Z, Cardona C, Dao P, Crossley B, Hietala S, Boyce W. Inability of real-time reverse transcriptase PCR assay to detect subtype H7 avian influenza viruses isolated from wild birds. J Clin Microbiol. 2008;46:1844–6. DOI: 10.1128/JCM.02426-07
7. Zhou EM, Chan M, Heckert RA, Riva J, Cantin MF. Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. Avian Dis. 1998;42:517–22. DOI: 10.2307/1592678
8. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9.
9. Saitou N, Nei M. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4:406–25.
10. Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 1986;3:418–26.
11. Krauss S, Obert CA, Franks J, Walker D, Jones K, Seiler P, et al. Influenza in migratory birds and evidence of limited intercontinental virus exchange. PLoS Pathog. 2007;3:e167. DOI: 10.1371/journal.ppat.0030167
12. Parmley EJ, Bastien N, Booth T, Bowes V, Buck P, Breault A, et al. Wild bird influenza survey, Canada, 2005. Emerg Infect Dis. 2008;14:84–7. DOI: 10.3201/eid1401.061562
13. Pasick J, Handel K, Robinson J, Copps J, Ridd D, Hills K, et al. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. J Gen Virol. 2005;86:727–31. DOI: 10.1099/vir.0.80478-0

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Merkel Cell Polyomavirus DNA in Persons without Merkel Cell Carcinoma

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Merkel cell polyomavirus (MCPyV) DNA was detected in 88% of Merkel cell carcinomas in contrast to 16% of other skin tumors. MCPyV was also found in anogenital and oral samples (31%) and eyebrow hairs (50%) of HIV-positive men and in forehead swabs (62%) of healthy controls. MCPyV thus appears to be widespread.

Merkel cell polyomavirus (MCPyV) was recently discovered in Merkel cell carcinomas (MCC), rare but aggressive skin cancers (*1*). MCPyV DNA has been detected in the majority of MCC and less commonly in other skin tumors and healthy skin (*1*–*6*). To help determine if MCPyV might be widespread in the general population, we conducted a retrospective study and tested MCC as well as healthy and lesional skin and mucosa samples of immunocompetent and immunosuppressed persons without MCC for MCPyV-DNA.

The Study

All samples (n = 355) were analyzed by hot-start single-round LT3-PCR (sPCR) and nested LT1/M1M2-PCR (nPCR) by using primers described previously (*1*) (experimental details on DNA isolation, controls, and PCR conditions are available from U.W.). Because analytical sensitivities of sPCR and nPCR were 1,000 copies of cloned LT3-DNA and 10 copies of cloned LT1-DNA per assay, samples positive by both PCRs probably had higher viral loads than those positive only by nPCR. The sPCR- or nPCR-products of 19 MCC and 48 non-MCC samples were sequenced and were MCPyV specific.

MCPyV DNA was detectable in 30/34 (88%) MCC biopsies and in 5/5 (100%) MCC metastases by nPCR, and in 68% and 80%, respectively, by sPCR. MCPyV DNA was found by nPCR only in 1/13 (7.7%) whole blood samples of MCC-patients. The patient with MCPyV-positive blood had positive sPCR/nPCR results for MCC and positive nPCR results for a second sample taken from the previous MCC site. Of 5 further non-MCC biopsy samples from MCC patients, 1 skin sample from a patient with unspecific dermatitis was positive by nPCR.

MCPyV DNA was traceable only by nPCR in 10/61 (16%) biopsy samples of different non-MCC skin tumors and in 8/34 (24%) of perilesional, clinically, and histologically healthy skin samples from 56 immunocompetent patients (*7*) without MCC (Table 1). MCPyV DNA status was identical in 30/32 pairs of tumor and corresponding perilesional skin samples (negative/negative in 24, positive/positive in 6, divergent in 2 pairs). MCPyV was found significantly more often in MCC (n = 34) than in non-MCC skin tumors (n = 61) or perilesional skin biopsies (n = 34) ($p<0.001$; χ^2 test).

Mucosal samples were available from 79 HIV-infected men who have sex with men (HIV-MSM) (without MCC) participating in an anogenital dysplasia/human papillomavirus (HPV) screening program (*8*). MCPyV DNA was detectable in 37/120 (31%) of all mucosal (anal, penile, oral) samples by nPCR and in 10/120 (8%) by sPCR (Table 2). In anal samples, MCPyV DNA positivity was lowest in anal cancer tissues (14% by nPCR), followed by dysplasias (26%), swabs with normal cytology (30%), and benign lesions (33%). Similar values were found for penile samples; 29% of dysplasias, 33% of benign lesions, and 50% of normal swabs were MCPyV DNA positive. In oral samples, MCPyV DNA was detected in 39% of normal swabs, in 0% of benign lesions, and in 50% of carcinomas in situ. MCPyV DNA positivity was not associated with the presence of mucosal premalignant and malignant lesions (p = 0.597; n = 120; 1-sided analysis of variance test), in contrast

Table 1. MCPyV DNA in biopsies of immunocompetent patients without MCC*

Histologic diagnosis	No. samples	No. (%) MCPyV-positive by nested PCR
Papilloma/wart	4	0 (0)
Actinic keratosis	7	0 (0)
Keratoacanthoma	7	3 (43)
Squamous cell carcinoma	6	1 (17)
Bowen disease/carcinoma	4	1 (25)
Basal cell carcinoma	21	3 (14)
Malignant melanoma	12	2 (17)
All skin tumors	61	10 (16)
Perilesional healthy skin	34	8 (24)

*MCPyV, Merkel cell polyomavirus; MCC, Merkel cell carcinoma. All samples shown were negative for Merkel cell polyomavirus in single-round PCR. Six MCPyV-positive perilesional samples had an MCPyV-positive lesional counterpart (3 basal cell carcinomas, 2 keratoacanthomas, 1 squamous cell carcinoma); 1 MCPyV-positive perilesional biopsy had a MCPyV-negative counterpart (basal cell carcinoma); and of 1 MCPyV-positive perilesional biopsy the lesional counterpart was not available. For origin of samples see (*7*).

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DOI: 10.3201/eid1412.080470

Table 2. MCPyV DNA and human papillomavirus DNA in samples of HIV-positive men without Merkel cell carcinoma*

Diagnosis	No. samples	No. (%) MCPyV-positive by nPCR	No. (%) MCPyV-positive by sPCR	No. (%) HPV-positive	No. (%) HR-HPV–positive†
Normal‡ anogenital/oral swabs	49	18 (37)	7 (14)	32 (65)	31 (63)
CD4 ≤200/μL	18	6 (33)	3 (17)	13 (72)	13 (72)
CD4 >200/μL	31	12 (39)	4 (13)	19 (61)	18 (58)
Anal	20	6 (30)	3 (15)	20 (100)	20 (100)
Penile	6	3 (50)	3 (50)	4 (67)	3 (50)
Oral	23	9 (39)	1 (4)	8 (35)	8 (35)
Benign papilloma/acanthoma	21	6 (29)	2 (10)	20 (95)	10 (48)
Anal	12	4 (33)	2 (17)	12 (100)	5 (42)
Penile	6	2 (33)	0 (0)	6 (100)	2 (33)
Oral	3	0 (0)	0 (0)	2 (67)	3 (100)
Dysplasia/carcinoma in situ	43	12 (28)	1 (2)	41 (95)	38 (88)
Anal intraepithelial neoplasia	27	7 (26)	1 (4)	26 (96)	26 (96)
Penile intraepithelial neoplasia	14	4 (29)	0 (0)	13 (93)	12 (86)
Oral carcinoma in situ	2	1 (50)	0 (0)	2 (100)	0 (0)
Anal cancer	7	1 (14)	0 (0)	7 (100)	7 (100)
All anal, penile, or oral samples	120	37 (31)	10 (8)	100 (83)	86 (72)
CD4 ≤200/μL	45	13 (29)	5 (11)	38 (84)	36 (80)
CD4 >200/μL	75	24 (32)	5 (7)	62 (83)	50 (67)
Plucked eyebrow hairs	14	7 (50)	5 (36)	14 (100)	0 (0)
Cerebrospinal fluid	7	0 (0)	0 (0)	ND	ND

*MCPyV, Merkel cell polyomavirus; nPCR, nested PCR; sPCR, single-round PCR; HPV, human papillomavirus; HR, high-risk alpha-HPV. All samples were tested for beta-HPV. Eyebrow hairs were additionally tested for alpha-HPV, and all were positive; 2 were additionally low-risk alpha-HPV–positive. Alpha- and beta-HPV DNA were determined by PCRs as previously described (*8*).
†HR types comprise HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82. For details of the patient-collective see (*8*).
‡Normal swabs were those negative for intraepithelial lesion or malignancy.

to positivity for high-risk (HR)-alpha-HPV, the established etiologic agents of these lesions ($p = 0.001$; $n = 120$) (Table 2). MCPyV DNA positivity was not significantly different in mucosal samples from HIV-MSM with CD4 counts below or above 200/μL (29% vs. 32%; $p = 0.839$; $n = 120$; χ^2 test). For HR-HPV, a trend for a higher detection rate in patients with CD4 counts <200/μL could be observed (80% vs. 67%; $p = 0.145$; $n = 120$) (Table 2). In 7 cerebrospinal fluid samples from HIV-MSM with central nervous system problems, MCPyV DNA was not detected.

In an immunodeficient patient with WILD syndrome (warts, immunodeficiency, lymphedema, anogenital dysplasia) (*9*), MCPyV DNA was found by nPCR and sPCR on the abdominal, thigh, perianal, and vulvar skin, and by nPCR in the vagina, cervix, and intraanal canal (20/27 swabs were nPCR- and 4/27 sPCR-positive). A whole blood sample and 5 papilloma biopsies were MCPyV DNA negative. MCPyV DNA was detected by nPCR in the cellular pellet but not in the supernatant of a urine sample of the patient with WILD syndrome. The presence of MCPyV DNA in the cellular pellet was probably caused by MCPyV- positive urogenital cells flushed into the urine.

MCPyV DNA was not found in 13 BKPyV DNA positive urine samples from 11 renal-transplant recipients without MCC. MCPyV DNA was detected by nPCR in 7/14 (50%) of plucked eyebrow hairs of 14 HIV-MSM and by sPCR in 5/14 (36%) (Table 2) as well as in eyebrow hairs of the patient with WILD syndrome (nPCR-positive). Skin swabs covering 20 cm^2 of the forehead were taken from 13 healthy immunocompetent male adults (*10*), and MCPyV DNA was detected by nPCR in 8/13 (62%) and by sPCR in 5/13 (38%).

Conclusions

Using nested PCR, we found MCPyV DNA in 88% of in samples from persons with MCC. In 68%, viral DNA load was high enough to be detectable by sPCR. This finding is similar to detection rates reported before (54%–89%) (*1*–*6*) and confirms the association of MCPyV with MCC.

The MCPyV positivity of 16% in non-MCC skin tumors was significantly lower than in MCC; MCPyV DNA was only detectable by nPCR, pointing to lower viral loads than in MCC. Similar to our results, MCPyV DNA has been found in 12.5% of basal cell carcinomas and viral load was 4-log lower than in MCC (*2*). In other studies, MCPyV DNA was detected in 13% of squamous cell carcinomas and only in 1 keratoacanthoma of 156 non-melanoma skin cancers (*4*,*6*). The relatively low detection rate of MCPyV in non-MCC skin tumors, similar to that in healthy, perilesional skin, suggests that MCPyV probably does not play a role in the development of non-MCC skin tumors.

HR-alpha-HPV induces anogenital dysplasia/cancer and HIV-MSM have a strongly increased risk for developing these lesions (*11*). In anogenital samples of HIV-MSM, MCPyV DNA was less common in premalignant and malignant lesion samples than in benign samples or samples

with normal cytology. Thus, it is unlikely that MCPyV plays a role in the development of anogenital dysplasia/cancer in HIV-MSM. In contrast to HR-alpha-HPV, MCPyV recovery was not increased in HIV-MSM with advanced immunodeficiency.

MCPyV DNA was detected only once in hematolymphoid tissue and never in donated blood (*5*,*12*). Similarly, we could not detect MCPyV DNA in 12/13 blood samples obtained from patients with MCC and in the blood sample of the patient with WILD syndrome, who was MCPyV positive in numerous other samples. MCPyV DNA was not found in BKPyV-positive urine samples from renal transplant recipients or in the cell-free urine supernatant of the patient with WILD syndrome.

In normal skin, MCPyV DNA has been identified before by PCR and Southern blot in 1/6 biopsies (*1*) but not in 15 samples when real-time PCR was used (*4*). Surprisingly, we found MCPyV DNA by sPCR in 38% and by nPCR in 62% of area-wide skin swabs from the forehead of healthy controls. Furthermore, MCPyV DNA was found in 14% and 37% of normal mucosa swabs of HIV-MSM by sPCR and nPCR, respectively. Since Merkel cells are found within the basal layer of the epidermis (*13*), it is unlikely that they are collected in surface-swabs. This observation suggests that the detected MCPyV DNA either represents cell-free virus that may have been produced in Merkel cells or virus in superficial keratinocytes. Thirty-six percent (sPCR) and 50% (nPCR) of eyebrow hairs of HIV-MSM carried MCPyV DNA. High concentrations of Merkel cells were described in the bulge region of hair follicles (*14*). Hair bulbs have been suggested as a reservoir for beta-HPVs (*15*), and this may also be true for MCPyV. Our data demonstrate a widespread distribution of MCPyV in normal skin, mucosa and hair bulbs, although MCPyV does not reach the magnitude found for ubiquitous beta-HPV (*10*,*15*). Our nonpopulation based data need to be confirmed in cross-sectional studies, but it is likely that MCPyV is prevalent in the general population.

Acknowledgments

We thank Monika Junk and Nabila Ristow for excellent technical assistance, Soenke Weissenborn for support in statistical analyses, and Martin Hufbauer for assistance in cloning of PCR products.

This work was supported by the German Federal Ministry of Education and Research grant no. 01 KI 0771 (TP7) and by the Center for Molecular Medicine of the University of Cologne.

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References

1. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008;319:1096–100. DOI: 10.1126/science.1152586
2. Becker JC, Houben R, Ugurel S, Trefzer U, Pfohler C, Schrama D. MC polyomavirus Is frequently present in Merkel cell carcinoma of European patients. J Invest Dermatol. 2009;129:248–50. DOI: 10.1038/jid.2008.198
3. Foulongne V, Kluger N, Dereure O, Brieu N, Guillot B, Segondy M. Merkel cell polyomavirus and Merkel cell carcinoma, France. Emerg Infect Dis. 2008;14:1491–3. DOI: 10.3201/eid1409.080651
4. Garneski KM, Warcola AH, Feng Q, Kiviat NB, Leonard JH, Nghiem P. Merkel cell polyomavirus is more frequently present in North American than Australian Merkel cell carcinoma tumors. J Invest Dermatol. 2009;129:246–8. DOI: 10.1038/jid.2008.229
5. Kassem A, Schopflin A, Diaz C, Weyers W, Stickeler E, Werner M, et al. Frequent detection of Merkel cell polyomavirus in human Merkel cell carcinomas and identification of a unique deletion in the VP1 gene. Cancer Res. 2008;68:5009–13. DOI: 10.1158/0008-5472.CAN-08-0949
6. Ridd K, Yu S, Bastian BC. The presence of polyomavirus in non-melanoma skin cancer in organ transplant recipients is rare. J Invest Dermatol. 2009;129:250–2. DOI: 10.1038/jid.2008.215
7. Weissenborn SJ, Nindl I, Purdie K, Harwood C, Proby C, Breuer J, et al. Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. J Invest Dermatol. 2005;125:93–7. DOI: 10.1111/j.0022-202X.2005.23733.x
8. Kreuter A, Brockmeyer NH, Weissenborn SJ, Gambichler T, Stucker M, Altmeyer P, et al. Penile intraepithelial neoplasia is frequent in HIV-positive men with anal dysplasia. J Invest Dermatol. 2008;128:2316–24. DOI: 10.1038/jid.2008.72
9. Kreuter A, Hochdorfer B, Brockmeyer NH, Altmeyer P, Pfister H, Wieland U. A human papillomavirus-associated disease with disseminated warts, depressed cell-mediated immunity, primary lymphedema, and anogenital dysplasia: WILD syndrome. Arch Dermatol. 2008;144:366–72. DOI: 10.1001/archderm.144.3.366
10. Weissenborn SJ, De Koning MN, Wieland U, Quint WG, Pfister HJ. Intrafamilial transmission and family-specific spectra of cutaneous beta-papillomaviruses. J Virol. 2009;83:811–6. DOI: 10.1128/JVI.01338-08
11. Palefsky J. Human papillomavirus-related tumors in HIV. Curr Opin Oncol. 2006;18:463–8. DOI: 10.1097/01.cco.0000239885.13537.36
12. Sharp CP, Norja P, Anthony I, Bell JE, Simmonds P. Reactivation and mutation of newly discovered WU, KI, and Merkel cell carcinoma polyomaviruses in immunosuppressed individuals. J Infect Dis. 2009;199:398–404. DOI: 10.1086/596062
13. Moll I, Moll R. Early development of human Merkel cells. Exp Dermatol. 1992;1:180–4. DOI: 10.1111/j.1600-0625.1992.tb00186.x
14. Narisawa Y, Hashimoto K, Nakamura Y, Kohda H. A high concentration of Merkel cells in the bulge prior to the attachment of the arrector pili muscle and the formation of the perifollicular nerve plexus in human fetal skin. Arch Dermatol Res. 1993;285:261–8. DOI: 10.1007/BF00371594
15. Boxman IL, Berkhout RJ, Mulder LH, Wolkers MC, Bouwes Bavinck JN, Vermeer BJ, et al. Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. J Invest Dermatol. 1997;108:712–5. DOI: 10.1111/1523-1747.ep12292090

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Population-based Surveillance for Hepatitis C Virus, United States, 2006–2007

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Surveillance for hepatitis C virus infection in 6 US sites identified 20,285 newly reported cases in 12 months (report rate 69 cases/100,000 population, range 25–108/100,000). Staff reviewed 4 laboratory reports per new case. Local surveillance data can document the effects of disease, support linkage to care, and help prevent secondary transmission.

Hepatitis C virus (HCV) infection is a serious public health problem in the United States and throughout the world. At least 80% of acute infections become chronic (*1*); an estimated 3.2 million persons in the United States alone have chronic HCV infection (*2*). In 2004, an HCV diagnosis was made in 936 of 100,000 outpatient visits for healthcare and in 143 of 100,000 hospital discharges (*3*). This is a chronic infection in which complications are manifested decades after the initial infection. Complications and costs associated with chronic HCV infection are anticipated to increase during 2010–2019 (*4*), because the incidence of new infections peaked from the late 1960s to early 1980s (*5*).

Although identifying persons with HCV infection, including asymptomatic persons, is challenging, the benefits for overall public health make it worthwhile. Infected persons can be referred to care (*6*), treated (if appropriate) (*7*), and counseled to prevent complications. The Centers for Disease Control and Prevention (CDC) and the Council of State and Territorial Epidemiologists recognized these benefits, and in 2003, recommended that past or present infections with HCV (hereafter referred to as HCV infection because most of these cases likely represent chronic rather than acute or resolved HCV infections) become a nationally reportable condition. Surveillance for acute non-A, non-B hepatitis, which was mostly HCV infection, has been performed in the United States since 1982, but in 2007, a total of 33 states also conducted surveillance for HCV infection and reported 133,520 cases to CDC; however, these data remain unpublished.

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DOI: 10.3201/eid1509.081050

The Study

Our study had 2 objectives. The first objective was to describe findings from 6 US state or county health departments that have been funded by CDC to perform enhanced surveillance for HCV infection. The second objective was to discuss the limitations and challenges of conducting population-based surveillance for HCV infection in the United States.

The sites where enhanced hepatitis surveillance was conducted during 2006–2007 were Colorado, Connecticut, Minnesota, New York (excluding New York City), and Oregon; Pinellas County, Florida, a sentinel counties (*8*) site, also contributed hepatitis C reports. The combined population under surveillance from the 5 states and 1 county was an estimated 29.3 million in 2007 (Table). In each of these jurisdictions, clinical laboratories are required to report positive results from HCV assays. For this analysis, a confirmed case of HCV infection was identified in any person who, from July 1, 2006 through June 30, 2007, had at least 1 of the following: 1) a positive result for an HCV recombinant immunoblot assay (RIBA), 2) a positive nucleic acid test (NAT) result for HCV RNA, 3) a documented HCV genotype, or 4) a positive result for a screening test for antibodies against HCV (anti-HCV) with a signal-to-cutoff (s:co) ratio predictive of a true positive result for the given assay.

Laboratories and providers continuously reported positive results for HCV markers (e.g., anti-HCV, RIBA, NAT, genotype) to state or local health departments. Health department staff checked patients' names and dates of birth from each report against a surveillance database to determine whether a case had been previously reported. Newly reported cases (i.e., previously not captured in the database of this jurisdiction) were entered into this database along with hepatitis test results. Health department staff investigated cases and collected basic demographic and clinical information to confirm the case definition and to epidemiologically describe the case. We calculated rates of newly reported cases by using denominators available from the 2007 population estimates from the US Bureau of the Census (www.census.gov/compendia/statab).

Table. Newly reported cases of past or present HCV infection in 6 US locations, July 1, 2006–June 30, 2007*†

Characteristic	Colorado	Connecticut	Minnesota	New York‡	Oregon	Pinellas County, Florida	Total
Estimated population, 2007	4,862,000	3,502,000	5,198,000	11,068,000	3,747,000	924,000	29,301,000
Year registry initiated	1993	1994	1998	2003	2005	1999	
Sex, no. (%)							
F	1,088 (36.5)	1,360 (35.9)	494 (37.6)	2,882 (30.4)	711 (34.9)	302 (45.4)	6,837 (33.8)
M	1,879 (63.0)	2,429 (64.1)	804 (61.2)	6,595 (69.5)	1,285 (63.0)	359 (54.0)	13,351 (66.0)
Unknown	16 (0.5)	3 (0.1)	15 (1.1)	15 (0.2)	44 (2.2)	4 (0.6)	53 (0.3)
Age group, y, no. (%)							
0–14	16 (0.5)	9 (0.2)	10 (0.8)	14 (0.2)	9 (0.4)	5 (0.8)	63 (0.3)
15–24	126 (4.2)	127 (3.4)	37 (2.8)	318 (3.4)	55 (2.7)	25 (3.8)	688 (3.4)
25–39	638 (21.4)	741 (19.5)	200 (15.2)	1,527 (16.1)	386 (18.9)	69 (10.4)	3,561 (17.6)
40–54	1,645 (55.2)	2,158 (56.9)	798 (60.8)	5,153 (54.3)	1,149 (56.3)	411 (61.8)	11,314 (56.0)
≥55	546 (18.3)	755 (19.9)	267 (20.3)	2,438 (25.7)	422 (20.7)	155 (23.3)	4,583 (22.7)
Unknown	12 (0.4)	2 (0.0)	1 (0.1)	42 (0.4)	19 (0.9)	0	76 (0.4)
Case criteria, no. (%)§							
Anti-HCV and supplemental test	593 (19.9)	1,520 (40.1)	520 (39.6)	3,763 (39.6)	203 (10.0)	0	6,599 (32.5)
RIBA	527 (17.7)	466 (12.3)	384 (29.3)	1,404 (14.8)	185 (9.1)	15 (2.3)	2,981 (14.7)
HCV RNA	1,245 (41.7)	1,984 (52.3)	928 (70.7)	5,831 (61.4)	818 (40.1)	28 (4.2)	10,834 (53.4)
Genotype	586 (19.6)	21 (0.6)	304 (23.2)	1,473 (15.5)	207 (10.2)	142 (21.4)	2,733 (13.5)
Anti-HCV and s:co ratio	1,859 (62.3)	1,352 (35.7)	253 (19.3)	4,709 (49.6)	905 (44.4)	521 (78.4)	9,599 (47.3)
Source of report, no. (%)							
Laboratory	2,561 (85.9)	3,792 (100.0)	878 (66.9)	8,252 (86.9)	1,923 (94.3)	592 (89.0)	17,998 (88.7)
Others, combined¶	422 (14.2)	0	435 (33.1)	1,240 (13.1)	117 (5.7)	73 (11.0)	2,287 (11.3)
Total reports	2,983	3,792	1,313	9,492	2,040	665	20,285
Report rate/100,000 population	61.4	108.3	25.3	85.8	54.4	71.9	69.2

*HCV, hepatitis C virus; RIBA, recombinant immunoblot assay; anti-HCV, antibodies against HCV; s:co, signal-to-cutoff.
†A confirmed case requires laboratory confirmation. Laboratory criteria consist of at least 1 of the following: a positive result for a HCV RIBA, a positive result for a nucleic acid test for HCV RNA, an HCV genotype, or enzyme immunoassay with detection of anti-HCV and a s:co ratio predictive of a true positive result for a particular assay (see www.cdc.gov/ncphi/disss/nndss/casedef/hepatitisccurrent.htm for the 2005 Council of State and Territorial Epidemiologists/Centers for Disease Control and Prevention case definition).
‡Excludes New York, New York.
§Cases could be reported with more than minimum laboratory criteria; totals add up to >100%.
¶Other sources of reports included private healthcare providers, facilities such as hospitals and outpatient clinics, and institutions such as prisons, blood banks, and drug treatment centers, among others.

Two supplemental assessments were conducted. The first assessment measured the number of laboratory reports associated with each new case. Staff at each site monitored a convenience sample of laboratory reports and measured the number excluded, reasons for exclusion, and the number that eventually were classified as newly reported cases. The second assessment determined the validity of basic epidemiologic information. For this task, CDC drew a random sample of 10 cases per site from among those reported during the 12-month reporting period (n = 60) and extracted the following variables: date of birth, county of residence, sex, race, and clinical test results associated with HCV infection. Surveillance staff contacted at least 1 healthcare provider to independently collect this information. We measured agreement between the information initially reported and the information collected during the validation using a κ statistic (*9*).

The 6 sites reported a total of 20,285 cases of confirmed HCV infection that were previously unreported in their respective jurisdictions (Table). Of these, 66% of case-patients were male and 56% were 40–54 years of age (men and women combined) (Table). More than half (52%) of the reports lacked information on race or ethnicity. Most cases (89%) were reported by clinical laboratories. The laboratory criterion most frequently reported was a positive result for HCV RNA (53%). The rate of new reports of past or present HCV infection was 69/100,000 population (range 25–108/100,000).

Sites monitored all incoming reports on average for 8 days (range 5–16 days). A total of 2,180 reports were received and, among these, 491 (23%, range 13%–52%) met the case definition and were considered newly reported cases; Oregon had the highest proportion of newly reported cases (52%) and the newest registry. The remaining reports fell into the following categories: already in the database (68%, range 30%–78%), lacking value for s:co ratio (5%, range 3%–13%), negative test results for an HCV marker (2%, range 1%–4%), or missing key demographic data (1%, range 0%–2%).

All cases were confirmed to meet the case definition. Agreement was high for age ($\kappa = 1.0$, $p<0.001$), sex ($\kappa = 0.96$; $p<0.001$), and county of residence ($\kappa = 1.0$; $p<0.001$); county data were missing for 6 (10%) cases.

Conclusions

We documented that for every 4 laboratory reports, ≈1 newly reported case of HCV infection was identified. The overall annual rate of new case reports was 69/100,000 population in 6 sites that were conducting enhanced surveillance. In the 4 states (Colorado, Connecticut, Minnesota, Oregon) for which comparable data were available, the number of newly reported cases of HCV infection was at least 4× the number of newly reported HIV infections in 2006 (*10*). The 1 county in Florida was not included in the comparison because no HIV data were available.

Two limitations must be mentioned. First, we do not know how many of the newly reported cases represent current infections. In the United States, 80% of prevalent anti-HCV–positive cases are HCV RNA positive (*2*); thus, most laboratory confirmed cases reported to surveillance are likely chronic infections, but could also represent acute or resolved infections. Electronic laboratory reporting is the most efficient way to identify potential cases (*11*), but because no current laboratory test can distinguish acute from chronic HCV infections, identification of acute-phase cases requires contacting the provider or patient to determine whether acute symptoms were present. Due to the high volume of reports received, this level of follow-up was not routinely conducted.

The second major limitation is that testing patterns in the community are unknown. Providers are inconsistent about eliciting risk factor information and about testing and referring patients to specialists (*12*). Patient access to care and structural factors in institutions (e.g., incentives and disincentives for testing at jails, prisons, and drug treatment programs) and in the community (e.g., screenings) also affect testing and, therefore, the reporting rate.

The greatest value of conducting surveillance for chronic HCV at the state and local level is to measure local frequency of disease. Local and state health departments share information such that changes of residence of cases within the state over time would not result in a duplicate case count. However, in aggregating these data at the national level, an infected person who moved from 1 state to another would likely trigger a new report in another state, thus resulting in an overestimate of the national prevalence. Therefore, as a coordinated surveillance system for chronic HCV is developed, a mechanism to prevent duplication of cases across states will need to be developed.

Many factors affect case reporting, such as, local public health reporting requirements, the sophistication and capacity of laboratories to electronically report de-duplicated positive test results, availability of health department staff to conduct investigations and follow-up on reports, time since registry was initiated, and the capacity of the system to maintain ongoing surveillance efforts. Without an understanding of these factors, interpreting the meaning of new HCV infection case reports is difficult.

Local health departments need chronic HCV infection surveillance to document effects of disease, identify persons in need of linkage to care, and prevent complications among persons infected (*13*). However, accurately collecting the necessary information is challenging for health departments, and we currently lack evidence that obtaining these data will result in a lower incidence of illness and death. A full assessment of the benefits and costs of conducting comprehensive surveillance for chronic HCV infection is overdue. Currently, the enhanced hepatitis surveillance sites are developing recommendations for best practices and plan to share methods and tools with all interested health departments. Future studies should evaluate what level of surveillance for chronic HCV is feasible and whether the prevention benefit is worth the effort.

Acknowledgments

We thank T. Bryant, K. Gerard, A Gamarra, A Sofair, S. Huie-White, N. Stabach, B. Lovely, C. Noonan-Toly, A. Fountain, G. Van Ness, A. Thomas, D. Daniels, and E. Din for their contributions to this study.

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References

1. Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, et al. The natural history of community-acquired hepatitis C in the United States. The Sentinel Counties Chronic Non-A, Non-B Hepatitis Study Team. N Engl J Med. 1992;327:1899–905.
2. Armstrong GL, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. Ann Intern Med. 2006;144:705–14.
3. Everhart JE. Viral hepatitis. In: Everhart JE, editor. The burden of digestive diseases in the United States. Washington: National Institute of Diabetes and Digestive and Kidney Diseases. Washington: Government Printing Office; 2008. NIH publication no. 09-6443. p. 13–23.
4. Wong JB, McQuillan GM, McHutchison JG, Poynard T. Estimating future hepatitis C morbidity, mortality, and costs in the United States. Am J Public Health. 2000;90:1562–9. DOI: 10.2105/AJPH.90.10.1562
5. Armstrong GL, Alter MJ, McQuillan GM, Margolis HS. The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. Hepatology. 2000;31:777–82. DOI: 10.1002/hep.510310332

6. Yawn BP, Gazzuola L, Wollan PC, Kim WR. Development and maintenance of a community-based hepatitis C registry. Am J Manag Care. 2002;8:253–61.
7. Strader DB, Wright T, Thomas DL, Seef LB. Diagnosis, management, and treatment of hepatitis C. Hepatology. 2004;39:1147–71. DOI: 10.1002/hep.20119
8. Bell BP, Shapiro C, Alter MJ, Moyer LA, Judson FN, Mottram K, et al. The diverse patterns of hepatitis A epidemiology in the United States: implications for vaccination strategies. J Infect Dis. 1998;178:1579–84. DOI: 10.1086/314518
9. Fleiss J. The measurement of interrater agreement. In: Statistical methods for rates and proportions, 2nd ed. New York: Wiley Interscience; 1981. p. 212–36.
10. Centers for Disease Control and Prevention. HIV/AIDS surveillance report 2007;18 [cited 2009 Jan 14]. Available from http://www.cdc.gov/hiv/topics/surveillance/resources/reports/2006report/default.htm
11. Centers for Disease Control and Prevention. Automated detection and reporting of notifiable diseases using electronic medical records versus passive surveillance—Massachusetts, June 2006–July 2007. MMWR Morb Mortal Wkly Rep. 2008;57:373–6.
12. Shehab TM, Sonnad SS, Lok AS. Management of hepatitis C patients by primary care physicians in the USA: results of a national survey. J Viral Hepat. 2001;8:377–83.DOI: 10.1046/j.1365-2893.2001.00310.x
13. Centers for Disease Control and Prevention. Recommendations for prevention and control of hepatitis C virus (HCV) infection and HCV-related chronic disease. MMWR Recomm Rep. 1998;47(RR-19):1–39.

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Absence of Detectable Replication of Human Bocavirus Species 2 in Respiratory Tract

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Human bocavirus (HBoV) commonly infects young children and is associated with respiratory disease; disease associations of the divergent HBoV-2 species are unknown. Frequent HBoV-2 detection in fecal samples indicated widespread circulation in the United Kingdom and Thailand, but its lack of detection among 6,524 respiratory samples indicates likely differences from HBoV-1 in tropism/pathogenesis.

Since its discovery in 2005 (*1*), human bocavirus (HBoV) has been the subject of intense investigation as a potential cause of human respiratory disease (*2*). In addition to respiratory tract and systemic infections, HBoV DNA sequences are frequently detected in fecal samples during primary infections (*3,4*), although a causative role in viral gastroenteritis has not been established (*5*–*7*). Other parvoviruses, including canine and bovine members of the genus *Bocavirus*, can replicate in the gastrointestinal tract and are often linked to enteric disease (*8,9*).

Until recently, published genetic analyses reported minimal sequence variability of HBoV strains; 2 genetic lineages differed in nucleotide sequence by only 2% in the virus protein 2 (VP2) gene (*10*). However, more divergent HBoV-like variants, provisionally designated as HBoV species 2 (HBoV-2), have been identified in fecal samples from children in Pakistan and the United Kingdom. These viruses show >20% nt sequence divergence (*11*). Published primer sequences for HBoV contain several mismatches with HBoV-2 sequences that may prevent their amplification (*11*). Thus, published surveys of HBoV prevalence likely report only HBoV-1. Therefore, HBoV-2 may represent an additional, currently undetected, agent in respiratory or enteric disease.

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DOI: 10.3201/eid1509.090394

The Study

To investigate HBoV-2, we developed new PCR-based detection methods for HBoV by using primer sets highly conserved between HBoV-1 and HBoV-2 and species-specific primers for HBoV-2. Large-scale screening of persons in the United Kingdom and Thailand was performed to compare virus detection frequencies in respiratory and fecal samples.

A total of 6,138 respiratory samples from 3,754 persons (2,018 male, 1,722 female, 14 sex unknown) during January 1, 2007–June 30, 2008, were obtained from the Specialist Virology Centre (Edinburgh, UK). Samples were not identified but epidemiologic and demographic information was retained (*12*,*13*). Samples comprised 3,065 nasopharyngeal swabs/aspirates (NPAs) and throat swabs (83%). A total of 386 NPAs were obtained from 386 persons (229 male, 154 female, 3 sex unknown) in Bangkok during February 16, 2006–July 20, 2008.

A total of 2,500 fecal samples were obtained from patients (1,093 male, 1,398 female, and 9 sex unknown) in Edinburgh predominantly with gastroenteritis or other enteric diseases referred for bacteriologic screening during March, June, and September 2008. A total of 530 fecal samples were obtained predominantly from children (179 boys and 138 girls) <5 years of age with diarrhea during July 12, 2007–July 25, 2008, and a control group without diarrhea (116 male, 96 female, 1 sex unknown) during March 4–December 2, 2007, in Bangkok.

DNA was extracted from 200-μL samples of pooled or individual specimens (respiratory samples, clarified fecal supernatant) into 40 μL Tris-EDTA buffer as described (*13*). Respiratory and fecal samples from Edinburgh were screened in pools of 10; both sample types from Bangkok were screened individually. Screening was performed by using nested primers conserved between HBoV-1 and HBoV-2 in the nucleoprotein (NP)–1 gene (universal primers: outer sense [position 2589 in DQ000496 st2 isolate (*1*)]: 5′-CCWATCGTCYTSYACTGCTTYGA-3′; outer antisense [2980]: 5′-TAGCYAAGTGTYTWBKGTACACA TYAT-3′); inner sense [2727]: 5′-RTKSTGYGGB TTCTAYTGGCA-3′; and inner antisense [2963]: 5′-TACACATCATCCCARTAAYWACAT-3′).

Amplication conditions were 94°C for 2 min and 35 cycles at 94°C for 18 s, 50°C for 21 s, and 72°C for 1.5 min. Amplicons were differentiated by digestion with *Rsa*I. Fragments were sized by agarose gel electrophoresis. All known HBoV-1 sequences contain an *Rsa*I site between nt 2772 and nt 2773, resulting in fragments of 46 bp and 91 bp; this site is absent in HBoV-2 (undigested amplicon length of 237 bp).

Each pool or sample was additionally screened by using HBoV2-specific primers located in the nonstructural (NS)–1 gene (outer sense [1484]: 5′-AACAGATGGGCAAGCAGAAC-3′; outer antisense [2031]: 5′-AGGACAAAGGTCTCCAAGAGG-3′; inner sense [1618]: 5′-AACGATTGCAGACAACGCCTTA TA-3′; and inner antisense [2019]: 5′-TCCAAGA GGAAATGAGTTTGG-3′; sites matching all known HBoV-2 variants and not matching HBoV-1 variants are underlined.) Amplification conditions were 95°C for 2 min; 5 cycles at 95°C for 45 s, 53°C for 1 min, and 72°C for min; and 35 cycles at 95°C for 30 s, 51°C for 30 s, and 72°C for 45 s. Positive pools of fecal samples from Edinburgh were divided and individual component samples were tested.

Respiratory and fecal samples from both centers were screened by using universal primers, and positive samples were digested with *Rsa*I. Undigested amplicons and some predicted HBoV-1 fragments (46 bp and 91 bp) were sequenced to confirm virus identity. All samples were additionally screened with HBoV-2–specific primers; 16 undigested samples were positive with HBoV-2–specific primers, and all samples identified as HBoV-1 were negative. Thus, species-specific primers enabled effective screening of HBoV-2 among samples with high frequencies of HBoV-1.

HBoV-positive fecal samples were generally restricted to children <5 years of age (25 from 30 infected children whose ages were known) (Figure, panel A; Table). Median age of children infected with HBoV-2 (7–12 months) was lower than that for those infected with HBoV-1 (1–2 years). Infections with HBoV-1 and HBoV-2 were observed at low frequencies in older persons (2 and 5 of 1,791 persons >35 years of age, respectively). For respiratory samples, HBoV-1 infections showed a similar peak incidence among children 1–2 years of age (Figure, panel B), similar to that observed for fecal samples. This age group was most frequently infected in our previous analyses of respiratory samples from Edinburgh (*12*). There were no differences in frequencies of HBoV-1 or HBoV-2 infection between male and female participants. Samples from Bangkok were divided into those from persons with diarrhea (327) and asymptomatic controls (213); detection of HBoV-1 and HBoV-2 was restricted to persons with diarrhea (n = 12 and 2, respectively).

In contrast to its frequent detection in fecal samples, HBoV-2 was not detected in >6,500 respiratory samples (Table). However, high frequencies of HBoV-1 were recorded (14% among children in Bangkok and 3.4% among children in Edinburgh); the group from Edinburgh contained a substantial number of older children (37% >5 years of age).

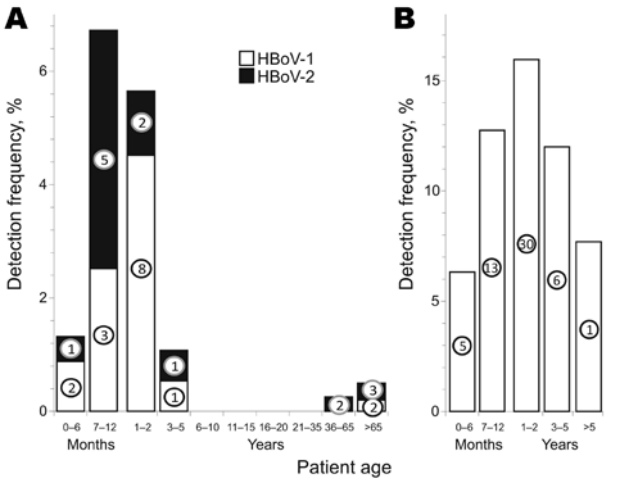

Figure. Age distribution of study participants with positive fecal (A) and respiratory (B) sample results for human bocavirus (HBoV), subdivided by HBoV species. Circles indicate numbers of positive samples in each category. Analysis of age distribution of persons with positive respiratory samples was restricted to samples from Bangkok, Thailand.

Conclusions

Four conclusions can be drawn from this study. First, HBoV-2 circulates in 3 widely separated areas (United Kingdom, Thailand, and Pakistan [*11*]) and is likely distributed globally. Second, infections with HBoV-2 show a pattern of infecting young children, most <1 year of age. Third, absence of HBoV-2 in respiratory samples suggests a different tissue tropism that may influence its transmission route and ability to infect systemically and establish persistence. Determining the biologic basis for such differences will be useful in understanding the pathogenesis of HBoV-1–related respiratory disease. Fourth, at a practical level, absence of HBoV-2 in respiratory samples indicates no likely role for this virus in respiratory disease. Thus, screening methods may be adequate for detecting HBoV-associated respiratory disease. Nevertheless, the unexpectedly diverse human bocavirus group may contain additional variants with potential etiologic roles in respiratory or other diseases.

Since this study was completed, evidence for an interspecies HBoV-1/-2 recombinant associated with acute gastroenteritis has been obtained; the structural gene region was most closely related to HBoV-2, and NS1/NP-1 grouping with HBoV-1 (*14*). Although this recombinant would have been identified as HBoV-1 by using typing assays described in the current study, sequence analysis of HBoV-1–positive samples in this study and our previous study of respiratory samples from Edinburgh and Bangkok (*12*,*15*) identified only HBoV-1 in the study population, consistent

Table. Frequency of human bocavirus species 1 and 2 in respiratory and fecal samples, United Kingdom and Thailand*

Sample type	Location	No. tested	Frequency, no. (%)	
			HBoV-1	HBoV-2
Fecal	Edinburgh, UK	2,500	6 (0.2)†	14 (0.6)†
	Bangkok, Thailand	530	10 (1.9)	2 (0.4)
Respiratory	Edinburgh, UK	6,138‡	67 (3.4)‡	0
	Bangkok, Thailand	386	55 (14.1)	0

*HBoV, human bocavirus.
†Frequency among children <5 y of age: HBoV-1, 1.4%; HBoV-2, 3.1%.
‡Tested in 241 pools composed of 2,294 samples. Frequencies were determined by using the Poisson formula, $f = \ln(f_0)$.

with all other analyses of this sample type worldwide. Nevertheless, future typing assays should analyze both VP1/2 and NS/NP-1 to ensure that this and potentially other interspecies recombinants are identified. Investigation of genetic diversity of this group and development of effective screening methods for variants of HBoV is required for studies of human disease.

Acknowledgments

We thank Gillian Fewster and the staff at the Microbiology Laboratory, Western General Hospital, Edinburgh, for providing fecal surveillance samples; and Elly Gaunt, Kate Templeton, and Carol Thomson for providing samples, data, and other virus testing results from the respiratory sample archive.

T.C. was supported by the Royal Golden Jubilee PhD Program; the Thailand Research Fund; the Center of Excellence in Clinical Virology, Chulalongkorn University; Biomedical Science, Graduate School, Chulalongkorn University; and the Commission on Higher Education, Ministry of Education, Thailand.

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References

1. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005;102:12891–6. DOI: 10.1073/pnas.0504666102
2. Schildgen O, Muller A, Allander T, Mackay IM, Volz S, Kupfer B, et al. Human bocavirus: passenger or pathogen in acute respiratory tract infections? Clin Microbiol Rev. 2008;21:291–304. DOI: 10.1128/CMR.00030-07
3. Vicente D, Cilla G, Montes M, Perez-Yarza EG, Perez-Trallero E. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis. 2007;13:636–7.
4. Neske F, Blessing K, Tollmann F, Schubert J, Rethwilm A, Kreth HW, et al. Real-time PCR for diagnosis of human bocavirus infections and phylogenetic analysis. J Clin Microbiol. 2007;45:2116–22. DOI: 10.1128/JCM.00027-07
5. Cheng WX, Jin Y, Duan ZJ, Xu ZQ, Qi HM, Zhang Q, et al. Human bocavirus in children hospitalized for acute gastroenteritis: a case-control study. Clin Infect Dis. 2008;47:161–7. DOI: 10.1086/589244
6. Chieochansin T, Thongmee C, Vimolket L, Theamboonlers A, Poovorawan Y. Human bocavirus infection in children with acute gastroenteritis and healthy controls. Jpn J Infect Dis. 2008;61:479–81.
7. Schildgen O, Muller A, Simon A. Human bocavirus and gastroenteritis. Emerg Infect Dis. 2007;13:1620–1.
8. Carmichael LE, Schlafer DH, Hashimoto A. Minute virus of canines (MVC, canine parvovirus type-1): pathogenicity for pups and seroprevalence estimate. J Vet Diagn Invest. 1994;6:165–74.
9. Durham PJ, Lax A, Johnson RH. Pathological and virological studies of experimental parvoviral enteritis in calves. Res Vet Sci. 1985;38:209–19.
10. Kesebir D, Vazquez M, Weibel C, Shapiro ED, Ferguson D, Landry ML, et al. Human bocavirus infection in young children in the United States: molecular epidemiological profile and clinical characteristics of a newly emerging respiratory virus. J Infect Dis. 2006;194:1276–82. DOI: 10.1086/508213
11. Kapoor A, Slikas E, Simmonds P, Chieochansin T, Naeem A, Shaukat S, et al. A newly identified bocavirus species in human stool. J Infect Dis. 2009;199:196–200. DOI: 10.1086/595831
12. Manning A, Russell V, Eastick KL, Hallam N, Templeton KE, Simmonds P. Epidemiological profile and clinical associations of human bocavirus and other human parvoviruses. J Infect Dis. 2006;194:1283–90. DOI: 10.1086/508219
13. Harvala H, Robertson I, McWilliam Leitch EC, Benschop K, Wolthers KC, Templeton K, et al. Epidemiology and clinical associations of human parechovirus respiratory infections. J Clin Microbiol. 2008;46:3446–53. DOI: 10.1128/JCM.01207-08
14. Arthur JL, Higgins GD, Davidson GP, Givney RC, Ratcliff RM. A novel bocavirus associated with acute gastroenteritis in Australian children. PLoS Pathog. 2009;5:e1000391. DOI: 10.1371/journal.ppat.1000391
15. Chieochansin T, Samransamruajkit R, Chutinimitkul S, Payungporn S, Hiranras T, Theamboonlers A, et al. Human bocavirus (HBoV) in Thailand: clinical manifestations in a hospitalized pediatric patient and molecular virus characterization. J Infect. 2008;56:137–42. DOI: 10.1016/j.jinf.2007.11.006

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Role of Rhinovirus C in Apparently Life-Threatening Events in Infants, Spain

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To assess whether infants hospitalized after an apparently life-threatening event had an associated respiratory virus infection, we analyzed nasopharyngeal aspirates from 16 patients. Nine of 11 infants with positive virus results were infected by rhinoviruses. We detected the new genogroup of rhinovirus C in 6 aspirates.

Human rhinovirus (HRV) is 1 of the most common agents associated with upper and lower respiratory tract infections in children and infants (*1*) and is a major trigger of asthma exacerbations (*2*). Recently, molecular methods have shown substantial phenotypic variation of HRV and identified a novel HRV genogroup provisionally named HRV-C (*3*). Severe asthma exacerbations in children have been associated with this new genogroup of rhinoviruses. Genogroup C could be resistant to a new candidate group of antipicornavirus drugs, including pleconaril (*4*).

Apparently life-threatening events (ALTEs) in infants are associated with bronchiolitis or infections in up to 6% of patients by diagnosis after hospital admission (*5*). We assessed the relation between ALTEs and respiratory virus infection in a secondary hospital in Spain.

The Study

Our study was part of a systematic prospective study to assess the epidemiology of respiratory virus infections in children admitted to the Severo Ochoa Hospital (Leganés, Madrid Province, Spain).We conducted a specific study to determine the incidence of respiratory virus infections in all infants admitted after ALTEs during November 2004–December 2008. An ALTE in a child <1 year of age was defined as an episode that is frightening to the observer and characterized by some combination of apnea, color change, marked change in muscle tone, choking, or gagging so the observer fears the infant has died (*6*).

Nasopharyngeal aspirate (NPA) specimens were acquired from each eligible patient at the time of hospital admission (on Monday–Friday). Samples were sent for virologic study to the Influenza and Respiratory Virus Laboratory (National Centre for Microbiology, Institute of Health Carlos III, Spain). Specimens were processed within 24 hours after collection.

Total nucleic acids were extracted from 200-μL aliquots by using a QIAamp MinElute Virus Spin Kit in a QIAcube automated extractor (QIAGEN, Valencia, CA, USA). Simple or multiplex reverse transcription–nested PCR assays (RT-PCR) previously described (*7–9*) were used to assess the virus diagnosis, including 16 respiratory viruses or groups of viruses. Degenerated primers for HRV and enteroviruses were designed between the 3′ end of the 5′ noncoding region (NCR) and the viral protein (VP) 4/VP2 polyprotein gene (TCIGGIARYTTCCASYACCA-ICC-3′ and CTGTGTTGAWACYTGAGCICCCA-3′). HRVs from positive samples were identified by sequencing and phylogenetic analysis of these sequences. Amplified products (about 500 bp, depending on HRV serotype) were purified and sequenced in both directions by using an automated ABI PRISM 377 model sequencer. Partial sequences of HRV have been submitted to GenBank (accession nos. FJ841954–FJ841957, FJ841959–FJ841961, EU697826, and EU697832). Appropriate precautions were implemented to avoid false-positive results by carryover contamination. Positive results were confirmed by testing a second aliquot of the sample stored at –70ºC.

Sixteen infants (8 of each sex) were enrolled in the study. All patients were <5 months of age (range 7 days–5 months, mean age 7.6 weeks, median 4 weeks). Twelve infants had rhinorrea, cough, and distress signs (Table). A total of 11 (69%) NPA specimens were positive for at least 1 viral agent. For 9 of these patients, positive results for HRV were confirmed, and for the other 2 patients, respiratory syncytial virus was detected.

Phylogenetic analyses of 9 sequences obtained from patients showed distribution of HRV in 3 clusters. Three sequences were included in previously characterized clades, defined by HRV group A (HRV-A, SO4923–EU697826) and B (HRV-B, SO3970–FJ841954 and SO4998–EU697832). Sequence from patient SO4923 had a low sequence similarity with the other serotypes of HRV-A. In contrast, sequences from patients SO3970 and SO4998 were closely related to HRV-35 and HRV-79, respectively. Six sequences were included in the third group corresponding to the new HRV-C: SO5854, SO6666, SO5797, SO6819, SO5986, SO6813- FJ841955-57 and FJ841959-61) (*3,10*) (Figure). Different genotypes (collectively called HRV-Cs) were identified in 6 NPA specimens

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DOI: 10.3201/eid1509.090453

Table. Characteristics of infants with ALTEs, Spain, November 2004–December 2008*

Laboratory no.	Sex/ age, wk	Admission date	Clinical signs	Discharge diagnosis	Virus	Prematurity
SO3970	M/4	2004 Nov	Cough, rhinorrea, loss of consciousness, flaccidity	Choking	HRV-B	No
SO4891	M/4	2005 Oct	Cyanosis, loss of consciousness, flaccidity	Cyanosis	No	Yes (35 wk)
SO4923	M/15	2005 Nov	Apnea, flushing	Cyanosis + URTI	HRV-A	Yes (36 wk)
SO4998	F/8	2005 Nov	Choking, flushing, distress	URTI + GERD	HRV-B	No
SO5260	F/9	2006 Apr	Cough, choking	GERD	No	No
SO5355	M/6	2006 Jun	Apnea, cyanosis	URTI + GERD	No	No
SO5529	F/4	2006 Nov	Apnea, congestion, cyanosis	Bronchiolitis + GERD	RSV	No
SO5749	M/24	2007 Mar	Choking, cyanosis	Wheezing	No	No
SO5797	F/6	2007 Mar	Apnea, flushing	Choking + GERD	HRV-C	No
SO5854	F/6	2007 Apr	Cough, rhinorrea, apnea	URTI + GERD	HRV-C	No
SO5896	M/13	2007 Sep	Apnea, rhinorrea	Bronchiolitis + GERD	HRV-C	No
SO6012	F/7	2007 Oct	Cough, distress, apnea	URTI	RSV-A	No
SO6666	M/4	2008 Oct	Cyanosis, choking	Choking	HRV-C	No
SO6813	F/6	2008 Nov	Apnea, flaccidity	Apnea + GERD	HRV-C	No
SO6819	M/6	2008 Nov	Cough, rhinorrea, flushing	URTI + GERD	HRV-C	No
SO6816	F/1	2009 Jan	Choking, flushing	Choking	No	No

*ALTEs, acute life-threatening events; URTI, upper respiratory tract infection; RSV, respiratory syncytial virus; HRV, human rhinovirus; EV, enterovirus; GERD, gastroesophageal reflux disease.

from children with ALTEs (67% of total HRV). Two received cardiopulmonary resuscitation at home; for these 2 patients, a respiratory syncytial virus and an HRV-C were identified. All 16 children survived.

Conclusions

The most common discharge diagnoses reported for ALTEs are gastroesophageal reflux disease (GERD), unknown causes, seizures, and lower respiratory tract infections (*11*). Our series suggests that ALTEs of previously unknown etiology could be related to HRV infections. Rhinovirus infections are known to be a major cause of illness and hospital admission for young children, particularly infants <2 years of age (*12*). Detection of viral genomes by nested RT-PCR in NPA specimens led us to analyze the effect of HRV infections in different clinical situations. Respiratory infections associated with HRV might play a major role in young infants, probably with few clinical signs, and might contribute to apnea as a first manifestation. GERD is the most frequent hospital discharge diagnosis in published series (*5,11*). For our patients, GERD also was the most frequent clinical diagnosis (9 patients), but for 7 of them, a respiratory virus was identified. We cannot conclude whether GERD is a risk factor for apnea or whether signs are so nonspecific that diagnoses could be confused.

Alternatively, the new HRV-C group could account for as many as a quarter or even half of HRV infections (*4,13*). In children, it has been associated with bronchiolitis, wheezing, and asthma exacerbations severe enough to require hospitalization; the percentage of these children with hypoxia was substantial (*13*). In a case–control study, Khetsuriani et al. (*4*) found HRV-C only in case-patients,

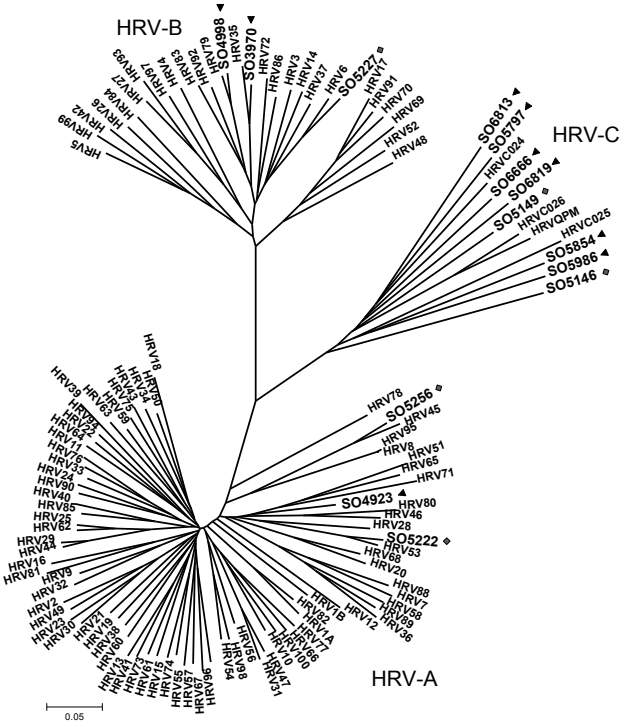

Figure. Phylogenetic analysis of 5′ noncoding region and viral protein (VP) 4/2 coding region of 9 human rhinoviruses (HRVs) identified in infants with apparently life-threatening events in Spain, November 2004–December 2008. Phylogeny of nucleotide sequences (≈492 bp) was reconstructed with neighbor-joining analysis by applying a Jukes-Cantor model; scale bar indicates nucleotide substitutions per site. Included for reference are sequences belonging to the novel genotype reported previously (QPM and 024, 025, 026 [*11*]) and all HRV-A and -B serotypes available in GenBank. Significant bootstrapping is indicated.

supporting the pathogenic role of this genogroup. They considered that HRV-C infections could be associated with more severe clinical manifestations than infections with other HRV genogroups A and B. These data could also support the role of HRV-C in infants with ALTEs found in this work.

Although we had no control group for our patients, we recently published a study of a cohort of 316 newborns up to 6 months of age tested weekly for respiratory diseases (mainly upper respiratory tract infections), coincident in age and time with our patients (*14*). HRV was present in 5 (3.6%) of 72 infants tested. Two viruses were genetically identified as HRV-C, demonstrating they form distinct genetic clusters, and no genetic similarity was obtained with the ALTE–related HRV-C viruses. In addition, a second group of asymptomatic children of different ages but in coincident epidemic seasons was studied. The group of children with HRV was substantially smaller than the group of children with respiratory disease (*15*).

Viral infections could play a major role in ALTEs. Rhinoviruses, especially HRV-C, could cause a respiratory infection with few symptoms in young infants and could trigger ALTEs in this age group. Therefore, HRVs and posterior genotyping should be included in studies of the etiology of ALTEs to help identify the true relevance of HRV-C infection to these episodes.

Acknowledgments

We thank Lola Lopez-Valero, Nieves Cruz, Monica Sánchez, and Ana Calderón for technical assistance.

This work was supported by grant PI060532 by Fondo de Investigaciones Sanitarias, Institute of Health Carlos III. Research on viral respiratory infections is carried out in collaboration with the Influenza and Respiratory Viruses Laboratory at the National Center of Microbiology (ISCIII) and supported by the Health Research Fund.

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References

1. Kusel MMH, Klerk NH, Holt PG, Kebadze T, Johnston SL, Sly P. Role of respiratory viruses in upper and lower respiratory tract illness in the first year of life. A birth cohort study. Pediatr Infect Dis J. 2006;25:680–6. DOI: 10.1097/01.inf.0000226912.88900.a3
2. Lemanske RF Jr, Jackson DJ, Gangnon RE, Evans MD, Li Z, Shult PA, et al. Rhinovirus illnesses during infancy predict subsequent childhood wheezing. J Allergy Clin Immunol. 2005;116:571–7. DOI: 10.1016/j.jaci.2005.06.024
3. Lamson D, Renwick N, Kapoor V, Liu Z, Palacios G, Ju J, et al. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004–2005. J Infect Dis. 2006;194:1398–402. DOI: 10.1086/508551
4. Khetsuriani N, Lu X, Teague WG, Kazerouni N, Aderson LJ, Erdman DD. Novel human rhinoviruses and exacerbation of asthma in children. Emerg Infect Dis. 2008;14:1793–6. DOI: 10.3201/eid1411.080386
5. Bonkowsky JL, Guenther E, Filloux FM, Srivastava R. Death, child abuse and adverse neurological outcome of infants after apparent life-threatening event. Pediatrics. 2008;122:125–31. DOI: 10.1542/peds.2007-3376
6. National Institutes of Health Consensus Development Conference on Infantile Apnea and Home Monitoring, Sep 29 to Oct 1, 1986. Pediatrics. 1987;79:292–9.
7. Pozo F, García-García ML, Calvo C, Cuesta I, Pérez-Breña P, Casas I. High incidence of human bocavirus infection in children in Spain. J Clin Virol. 2007;40:224–8. DOI: 10.1016/j.jcv.2007.08.010
8. Coiras MT, Aguilar JC, Garcia ML, Casas I, Perez-Brena P. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J Med Virol. 2004;72:484-95.
9. López-Huertas MR, Casas I, Acosta-Herrera B, Garcia ML, Coiras MT, Pérez-Breña P. Two RT-PCR based assays to detect human metapneumovirus in nasopharyngeal aspirates. J Virol Methods. 2005;129:1–7. DOI: 10.1016/j.jviromet.2005.05.004
10. Briese T, Renwick N, van den Berg M, Jarman R, Ghosh D, Köndgen S, et al. Role of rhinovirus in hospitalized infants with respiratory tract infections in Spain. Emerg Infect Dis. 2008;14:944–7. DOI: 10.3201/eid1406.080271
11. McGovern MC, Smith MB. Causes of apparent life threatening events in infants: a systematic review. Arch Dis Child. 2004;89:1043–8. DOI: 10.1136/adc.2003.031740
12. Calvo C, García-García ML, Blanco C, Pozo F, Casas I, Perez-Breña P. Rhole of rhinovirus in hospitalized infants with respiratory tract disease in Spain. Pediatr Infect Dis J. 2007;26:904–8. DOI: 10.1097/INF.0b013e31812e52e6
13. Miller EK, Edwards KM, Weinberg GA, Iwane MK, Griffin MR, Hall CB et al. A novel group of rhinoviruses is associated with asthma hospitalizations. J Allergy Clin Immunol. 2009;123:105–6.
14. Bueno Campaña M, Calvo Rey C, Vázquez Alvarez MC, Parra Cuadrado E, Molina Amores A, Rodrigo García G, et al. Infecciones virales de vías respiratorias en los primeros 6 meses de vida. An Pediatr (Barc). 2008;69:400–5. DOI: 10.1157/13127993
15. García-García ML, Calvo C, Pozo F, Pérez-Breña P, Quevedo S, Bracamonte T, et al. Human bocavirus detection in nasopharyngeal aspirates of children without clinical symptoms of respiratory infection. Pediatr Infect Dis J. 2008;27:358–60. DOI: 10.1097/INF.0b013e3181626d2a

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Saffold Cardiovirus in Children with Acute Gastroenteritis, Beijing, China

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To understand Saffold cardiovirus (SAFV) distribution, prevalence, and clinical relevance in China, we retrospectively studied SAFV in children with acute gastroenteritis and found SAFV in 12 (3.2%) of 373. Sequence homology of virus protein 1 genes suggested these strains belong to the SAFV-1 sublineage. SAFVs were found in samples positive for other diarrhea-causing viruses.

Recently, a new virus, provisionally named Saffold virus (SAFV), was recovered in the United States from a fecal sample from an 8-month-old girl with fever of unknown origin (*1*). The newly identified virus was classified under the genus *Cardiovirus,* family *Picornaviradae.* The 2 known species of the genus *Cardiovirus, encephalomyocarditis viruses* and *Theiler viruses* (*2*), are known to be pathogenic in several animal species and in humans (*3*–*6*). SAFV is genetically related to *Theiler viruses* and is believed to constitute a novel cardiovirus species (*1*,*7*). SAFV has been detected in children with enteric or respiratory tract infections in the United States, Canada, Brazil, Germany, Pakistan, and Afghanistan (*1*,*8*–*11*). However, the worldwide distribution of SAFV and its clinical significance remain unclear. To understand SAFV distribution, prevalence, and clinical relevance in the People's Republic of China, we conducted a retrospective study by screening for SAFV in children with acute gastroenteritis.

The Study

From March 2006 through November 2007, fecal samples were collected from 373 pediatric outpatients at Beijing Children's Hospital in a prospective study on viral etiology of diarrhea. The ages of patients ranged from 1 month to 13 years (mean age 11.7 months, median age 9.0 months). Gastroenteritis was defined as acute watery diarrhea accompanied by other clinical signs and symptoms such as fever, nausea, and vomiting. No patient had any apparent clinical respiratory signs or symptoms.

We diluted these previously collected fecal specimens to 10% (wt/vol) with phosphate-buffered saline (pH 7.2) and removed cellular debris by centrifugation (2,500× *g* for 5 min). Virus nucleic acids were extracted by using the NucliSens miniMAG platform according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). SAFV RNA was detected in the samples by nested reverse transcription–PCR (RT-PCR) that used primers targeting the 5′ untranslated region (UTR), which generated a 540-bp amplicon (*9*). Primers cardioVP1-1F/4R and cardioVP1-2F/3R, which span the virus protein 1 (VP1) gene, were used for a nested RT-PCR to amplify the VP1 gene (about 910 bp) as previously described (*10*). Because VP1 genes of 2 SAFV-positive samples could not be amplified in this way, a newly designed primer pair (cardioVP1Fn: TCAGAATGCCAATCTCCCCAAC and cardioVP1Rn: AAAGGTCCACCCGATACATTGA) was used in combination with cardioVP1-2F/3R to amplify the VP1 gene based on the sequences obtained from our positive samples. Conditions for first- and second-round PCR were 94°C for 3 min, followed by 40 cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 90 sec, and a final 10-min cycle at 72°C. All positive PCR amplicons were verified by sequencing after being cloned into the pMD18T vector (Takara Bio Inc., Dalian, China). Three positive clones were randomly selected for parallel sequencing. Each sample was screened by PCR for enteric adenovirus, astrovirus, noroviruses, sapovirus, and human bocavirus by using PCR (*12*,*13*) and for group A rotaviruses by using the Rotavirus ELISA Diagnostic Kit (Lanzhou Institute for Biological Products, Lanzhou, China). To characterize the nucleotide sequences obtained from this study, we analyzed the 5′ UTR and VP1 genes of all SAFV isolates to determine the extent of homology among the genes and those documented in the GenBank database by using MEGA software (*14*).

SAFV RNA was detected in 12 (3.2%) of 373 fecal specimens by using RT-PCR with primers targeting the 5′ UTR gene. Of these 12 positive specimens, 5 were collected from boys and 7 from girls. The ages of SAFV-positive patients ranged from 1 month to 3 years (mean age 12.3 months, median age 9.5 months). This age distribution is similar to that reported by Chiu et al., who detected SAFV mainly in younger children (*10*).

Co-infections with other viruses, including rotavirus (7/11) and norovirus (5/11), were detected in 11/12 SAFV-positive specimens (Table). The prevalence of rotavirus and norovirus infection in this sample pool was 59.5%

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DOI: 10.3201/eid1509.081531

Table. Fecal samples positive for Saffold cardiovirus, Beijing, People's Republic of China, March 2006–November 2007*

Sample no.	Patient sex/age, mo	Codetected virus
GL311	F/10	Rotavirus
GL317	F/20	None
GL328	M/24	Rotavirus
GL341	F/36	Rotavirus
GL352	F/6	Norovirus
GL361	F/11	Norovirus
GL362	M/1	Norovirus
GL365	M/10	Rotavirus
GL368	F/7	Rotavirus
GL371	F/9	Norovirus
GL376	M/8	Rotavirus
GL377	M/5	Rotavirus + norovirus

*All patients were from urban areas of Beijing and had acute gastroenteritis.

(222/373) and 12.1% (45/373), respectively. SAFV-positive samples were found only in the last month of the 18-month study period, November 2007.

To assess the sequence variations of SAFV strains detected in this study, we analyzed an 825-bp cDNA fragment (GenBank accession nos. FJ464766–FJ464777) corresponding to the VP1 gene of the 12 SAFV strains. Previous studies have demonstrated the existence of 8 distinct phylogenetic sublineages of SAFV on the basis of the homology of P1 and VP1 genes (*9*,*11*). However, all strains identified in this study appeared to be the SAFV-1 sublineage (Figure), and they showed 99.2%–100% homology in nucleotide sequences and 98.5%–100% homology in the amino acid sequence of the VP1 gene. The identity of the VP1 amino acid sequences among these strains and the prototype SAFV (EF165067) was as high as 98.1%–98.9%. Multiple-alignment analysis showed that the amino acid sequence identity of VP1 among all available isolates varied from 62.3% to 100% (online Appendix Table, available from www.cdc.gov/EID/content/15/8/1509-appT.htm), indicating the global diversity of the SAFV strains from different geographic locations.

Conclusions

This retrospective study showed that 12 (3.2%) of 373 children with acute gastroenteritis in Beijing were SAFV-positive, indicating the prevalence of recently characterized SAFV in China and providing evidence of global distribution of SAFV. Although SAFVs have been detected in samples collected from enteric and respiratory tracts, the clinical role for SAFV is still unclear. In this study, 11 of 12 SAFV-positive samples were co-infected with at least 1 known diarrhea-causing virus, such as rotavirus or norovirus. Therefore, we cannot conclusively state that SAFV is responsible for gastroenteritis (*8*–*11*).

That all SAFV-positive samples were collected in November 2007 suggests a possible seasonal outbreak. However, because of insufficient background information from outpatients, whether the rate of SAFV detection peaks in a single month or whether it indicates a seasonal outbreak is unclear. Further investigations are necessary. Nevertheless, our finding suggests a potential epidemic of SAFV during the cold season (*9*).

In the samples used for this study, we detected SAFV-1 only, no other sublineages (*9*,*11*). Although isolated 20 years ago in San Diego, California, USA, the SAFV-1 sequence was not published until 2007 because of the progress of molecular techniques for unknown genome cloning (*1*). The reason for this 20-year hiatus of SAFV-1 in the United States and the occurrence of the same genotype in China in 2007 is unclear. It can be attributed to a geographic variation of an SAFV epidemic in the world. Currently, 8 SAFV sublineages have been identified in different areas: SAFV-2 and SAFV-3 have been detected in North and South America (United States and Brazil) and Europe (*9,*

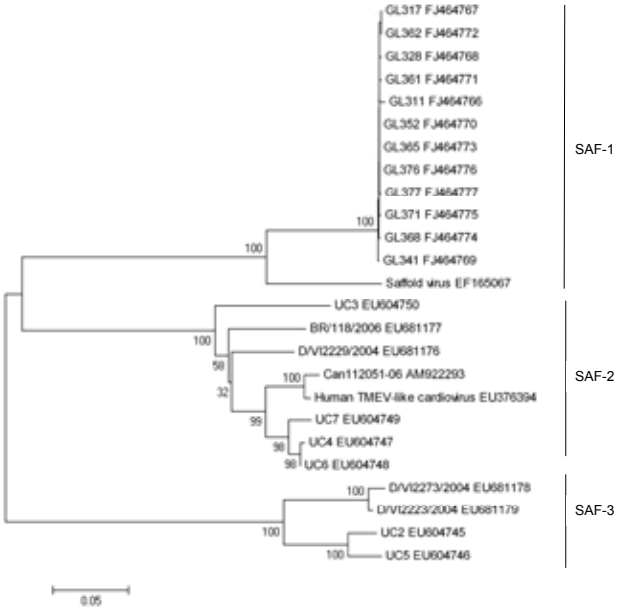

Figure. Phylogenetic analysis of nucleotide sequences of the virus protein 1 (VP1) gene of Saffold cardiovirus. The tree was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) software version and the neighbor-joining algorithm with kimura-2 parameters (*14*). The analysis included human Theiler murine encephalomyelitis virus (TMEV)–like cardiovirus. TMEV-like cardiovirus sequences (GenBank accession no. EU376394) and the previously reported SAFV sequences including the prototype SAFV, U2-U7, Can112051-06, BR/118/2006, D/VI2229/2004, D/VI2223/2004, and D/VI2273/2004 (GenBank accession nos. EF165067, NC009448, EU604745-EU604750, AM922293, and EU681176-EU681179) as references. Because the sequences of SAFV-4 to SAFV-8 are not available, they are not included in the phylogenetic tree. Each strain from this study is indicated by a specific identification code (GL) followed by the patient number (GL311, GL317, GL328, GL341, GL352, GL361, GL362, GL365, GL368, GL371, GL376, and GL377) and its GenBank accession number. Scale bar indicates nucleotide substitutions per site.

10), and SAFV-2 to SAFV-8 have been detected in South Asia (*11*). SAFV-1 may be the dominant sublineage in circulation in Beijing and may have escaped detection until the current investigation. The dominant SAFV sublineages may be changing over time in a certain geographic area. An SAFV-1 endemic to the United States in the1980s may have been subsequently replaced by SAFV-2 and SAFV-3, whereas SAFV-1 has now become dominant in Beijing. Because the existence of these human Theiler-like viruses was unknown before 2007, comprehensive global investigations of the prevalence and diversity of SAFV, especially studies based on samples collected over the previous years, will be helpful in providing further insights into SAFV origin, sublineage, and distribution.

Acknowledgments

We thank Li Guo for her assistance with verifying PCR results. We also thank Yongjun Li for his assistance with sequence analysis.

This study was supported in part by grants from National Science Research Megaproject against Major Infectious Diseases in China (2009ZX10004-206); Fondation Mérieux; and Institute of Pathogen Biology, Chinese Academy of Medical Sciences.

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References

1. Jones MS, Lukashov VV, Ganac RD, Schnurr DP. Discovery of a novel human picornavirus in a stool sample from a pediatric patient presenting with fever of unknown origin. J Clin Microbiol. 2007;45:2144–50. DOI: 10.1128/JCM.00174-07
2. Stanway G, Brown F, Christian P, Hovi T, Hyypiä T, King AMQ, et al. Family *Picornaviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses. London: Elsevier/Academic Press; 2005. p. 757–78.
3. LaRue R, Myers S, Brewer L, Shaw DP, Brown C, Seal BS, et al. A wild-type porcine encephalomyocarditis virus containing a short poly (C) tract is pathogenic to mice, pigs, and cynomolgus macaques. J Virol. 2003;77:9136–46. DOI: 10.1128/JVI.77.17.9136-9146.2003
4. Knowles NJ, Dickinson ND, Wilsden G, Carra E, Brocchi E, De Simone F. Molecular analysis of encephalomyocarditis viruses isolated from pigs and rodents in Italy. Virus Res. 1998;57:53–62. DOI: 10.1016/S0168-1702(98)00081-1
5. Grobler DG, Raath JP, Braak LE, Keet DF, Gerdes GH, Barnard BJ, et al. An outbreak of encephalomyocarditis-virus infection in free-ranging African elephants in the Kruger National Park. Onderstepoort J Vet Res. 1995;62:97–108.
6. Kirkland PD, Gleeson AB, Hawkes RA, Naim HM, Boughton CR. Human infection with encephalomyocarditis virus in New South Wales. Med J Aust. 1989;151:176–8.
7. Liang Z, Kumar AS, Jones MS, Knowles NJ, Lipton HL. Phylogenetic analysis of the species Theilovirus: emerging murine and human pathogens. J Virol. 2008;82:11545–54. DOI: 10.1128/JVI.01160-08
8. Abed Y, Boivin G. New Saffold cardioviruses in 3 children, Canada. Emerg Infect Dis. 2008;14:834–6. DOI: 10.3201/eid1405.071675
9. Drexler JF, Luna LK, Stöcker A, Almeida PS, Ribeiro TC, Petersen N, et al. Circulation of 3 lineages of a novel Saffold cardiovirus in humans. Emerg Infect Dis. 2008;14:1398–405 DOI: 10.3201/eid1409.080570
10. Chiu CY, Greninger AL, Kanada K, Kwok T, Fischer KF, Runckel C, et al. Identification of cardioviruses related to Theiler's murine encephalomyelitis virus in human infections. Proc Natl Acad Sci U S A. 2008;105:14124–9. DOI: 10.1073/pnas.0805968105
11. Blinkova O, Kapoor A, Victoria J, Jones M, Wolfe N, Naeem A, et al. Cardioviruses are genetically diverse and common enteric infections in south Asian children. J Virol. 2009;83:4631–41. DOI: 10.1128/JVI.02085-08
12. Rohayem J, Berger S, Juretzek T, Herchenröder O, Mogel M, Poppe M, et al. A simple and rapid single-step multiplex RT-PCR to detect norovirus, astrovirus and adenovirus in clinical stool samples. J Virol Methods. 2004;118:49–59. DOI: 10.1016/j.jviromet.2004.01.016
13. Chung JY, Han TH, Kim CK, Kim SW. Bocavirus infection in hospitalized children, South Korea. Emerg Infect Dis. 2006;12:1254–6.
14. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092

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Human Infection with G12 Rotaviruses, Germany

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Rotavirus group A G12 genotypes were detected in 3 (1.5%) of 198 stool samples positive for human rotavirus. G12P[6] was present in 2 samples, and a mixed G3G12P[8] was found in 1 sample. Phylogenetic analysis of complete open reading frames of all 11 genomic RNA segments proved their Wa-like genogroup affiliation.

Rotaviruses are worldwide enteric pathogens in humans and animals. Most prevalent human strains in Europe are group A rotaviruses with genotypes G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] (*1*,*2*). Oral live attenuated vaccines were licensed in 2006 in Germany after they were found to be efficient and safe. Monovalent Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) contains a G1P[8] strain, whereas RotaTeq (Merck and Co., West Point, PA, USA) contains 5 bovine-human reassortants representing genotypes G1–4 in association with P[5] and G6P[8] (*3*,*4*). To evaluate rotavirus vaccine efficacy and possible escape of genotypes from host immunity, postmarketing monitoring of circulating wildtype rotaviruses is necessary. That G12, which was detected 1987 in the Philippines, will be a predominant genotype in the future has been assumed. In recent years, a growing number of countries worldwide have reported the occurrence of G12, both sporadically and as a genotype of notable incidence (*5*).

The Study

A total of 2,752 stool specimens were collected in 2008 from inpatients with diarrhea at Leipzig University Hospital. The samples were derived from 1,804 patients, of whom 715 were <6 years of age. Several aliquots of a 10% stool suspension in phosphate-buffered saline were prepared from each specimen. One aliquot was screened on rotavirus group A antigen by IDEIA (Dako Ltd, Ely, UK). RNA of antigen-positive samples was extracted from a second aliquot by NucliSens easyMAG system (bioMérieux, Boxtel, the Netherlands). Rotavirus gene segments coding for structural viral proteins (VP) 1, VP2, and VP3 were amplified by reverse transcription–PCR with consensus primers (Metabion, Martinsried, Germany): VP1-F 5′-GGCTATTAAAGCTGTACAATG-3′ (nt 1–21), VP1-R 5′-GGTCACATCTAAGCACTC-3′ (nt_3302–3285), VP2-F 5′-GGCTATTAAAGGCTCAAT-3′ (nt 1–18), VP2-R 5′-GGTCATATCTCCACAGTGG-3′ (nt 2717–2699), VP3-F 5′-GGCTATTAAAGCAATACTAG-3′ (nt 1–20), VP3-R 5′-GGTCACATCATGACTAGT-3′ (nt 2591–2574), and the other gene segments with primers described elsewhere (*6*–*8*). In the case of gene segments with short untranslated regions, primer ligation and reverse transcription steps were performed as described by Lambden et al. (*9*). Their primer 2 and gene-specific primers were used in subsequent PCRs to determine entire open reading frames.

Amplicons were gel purified by using Wizard SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany) and sequenced by PCR primers and internal primers with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). To separate amplicons of mixed infections, the amplicons were cloned into pCRII-TOPO vector and transformed into *Escherichia coli* (Invitrogen, Carlsbad CA, USA). Plasmids were purified by QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and sequenced with M13 primers (Invitrogen). Corrected chromatograms were assembled by using ContigExpress Module of VectorNTI Suite (Invitrogen). Full-length amino acid sequences were aligned by AlignX (a module of VectorNTI Suite). Phylogenetic analyses were conducted by MEGA version 4.0 software (www.megasoftware.net). Genetic distances were calculated by using the Poisson correction parameter. The dendrograms were constructed by the neighbor-joining method. Statistical support was assessed by bootstrapping with 1,000 replicates (*10*). The sequences of the 2 German G12 rotavirus strains were deposited in GenBank (Table).

Of samples from 1,804 patients, 198 (11%) were positive for rotavirus; of those with positive samples, 174 patients were <6 years of age. Genotyping showed G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], G12P[6], G1G9P[8], and G3G12P[8]. G12 rotavirus strains were detected in 3 stool specimens. Strain GER126–08 was derived from the specimen of a 10-year-old boy who had been admitted to the hospital on April 14 because of a first manifestation of type 1 diabetes. After 1 week, he was transferred to our pediatric ward where diarrhea and vomiting developed 3 days later. Rotavirus genotypes of concurrently hospitalized children on the same ward were distinct. G12 strain GER172–08 was found in samples of 2 bottle-fed young infants who had gastroenteritis: a 15-day-old boy on July 31 and a 30-day-old girl on August 11. There was no direct contact between the 3 patients, and none of the patients or their close family members had any migration background or recent travel abroad. They also had not been previously vaccinated against rotavirus.

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DOI: 10.3201/eid1509.090497

Table. GenBank accession numbers of both G12 strains of rotavirus found in Germany*

Gene segment	GER126-08	GER172-08
VP1	FJ747613	FJ747625
VP2	FJ747614	FJ747626
VP3	FJ747615	FJ747627
VP4	FJ747616	FJ747628
VP6	FJ747617	FJ747629
VP7	FJ747618, FJ747619	FJ747630
NSP1	FJ747620	FJ747631
NSP2	FJ747621	FJ747632
NSP3	FJ747622	FJ747633
NSP4	FJ747623	FJ747634
NSP5/6	FJ747624	FJ747635

*VP, viral protein; NSP, nonstructural protein.
†Sequences of both G12 and G3 genotypes were present in strain GER126-08.

Direct sequencing of gel-purified GER126-08 VP7 amplicons resulted in peak superpositions in sequencing gels; the sequencing of clones of this isolate showed a mixture of G3 and G12 genotypes. Contamination was excluded by a second RNA extraction and by comparing sequences to all G3 and G12 types detected in 2008, which were distinct. An amino acid alignment of VP7 G3 showed highest identity (97.5%) to G3 strains from Southeast Asia (data not shown). The G12 genotype belonged to G12-III lineage but was distinct from published full-length sequences, including the only European sequence from Belgium (Figure 1). Highest homology (97.8%) was shown in comparison to the Indian strain 14B2 (online Appendix Table, available from www.cdc.gov/EID/content/15/9/1512-appT.htm). Partial sequence data from European isolates showed no closer relationship, and G12 was not detected in 19 porcine rotaviruses from different piggeries of Saxony (data not shown). Amplification of VP1–4, VP6, and NSP1–5 by gene segment-specific consensus primers within conserved regions in the respective 5′ and 3′ ends was performed. No peak superposition occurred in sequencing of these amplicons, that is, only 1 variant of each genomic RNA segment could be detected. This finding indicates recent reassortment events. Although less likely, minor species of these 10 genomic RNA segments are not entirely excluded; they might have been missed in amplification or cloning and sequencing. The deduced amino acid sequences (online Appendix Table) signified a Wa-like genogroup virus (G3G12-P[8]-I1-R1-C1-M1-A1-N1-T1-E1-H1) (*11*,*12*). Its VP4 genomic RNA segment was phylogenetically related to those of Japanese G3 and G4 genotypes (Figure 2) and distinct from all other P[8] genotypes of this collection (data not shown). The origin of the associated genomic RNA segments from either a G3 or a G12 type rotavirus remains unclear, due to the lack of substantial numbers of available full-length sequences.

Rotavirus sequences in stool samples of the 2 newborns were identical. Full-length VP7 amino acid alignments of GER172-08 with published sequences showed 100% homology to G12-III strains ISO16 and ISO29 from India and to strains 04N245, 04N338, 05K021, 05K046, 05K066, and 05N138 from Nepal. The P[6] genotype of GER172-08 was not shared by other rotaviruses in this study, and it showed the highest homology to strains from Bangladesh and South Korea (Figure 2). Analysis of deduced amino acid sequences of all proteins showed a Wa-like genogroup affiliation (G12-P[6]-I1-R1-C1-M1-A1-N1-T1-E1-H1) (data not shown) (*11*,*12*). In a comparative analysis, GER126-08 is clearly distinct from GER172-08 (online Appendix Table).

Conclusions

Two distinct G12 rotaviruses with different P type associations were detected. The findings suggest that they

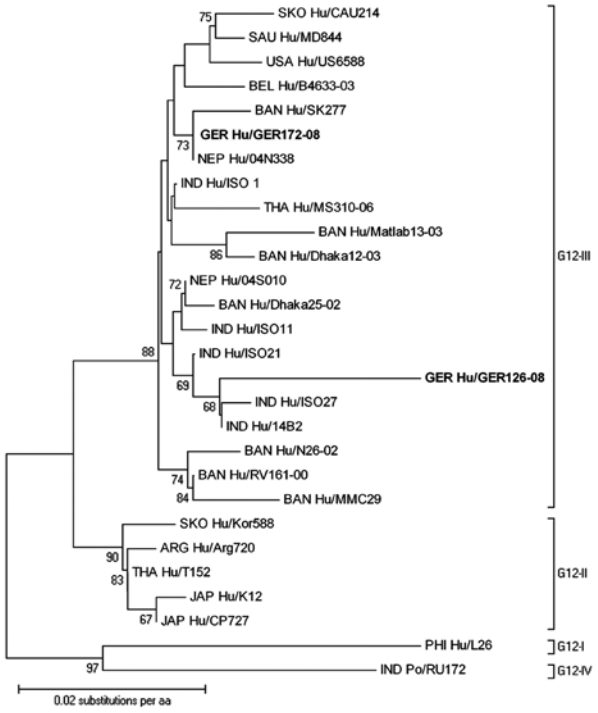

Figure 1. Phylogenetic dendrogram of viral protein 7 (VP7) of G12 rotavirus at the amino acid level. Bootstraps values (1,000 replicates) >65% are shown. The strain name is prefixed by the country of origin (ARG, Argentina; BAN, Bangladesh; BEL, Belgium; GER, Germany; IND, India; JAP, Japan; NEP, Nepal; PHI, Philippines; SAU, Saudi Arabia; SKO, South Korea; THA, Thailand; USA, United States of America) as well as the viral host (Hu, human, Po, porcine). **Boldface** indicates strains of this study. GenBank accession numbers of VP7 genes compared: 04N338 BAF64828, 04S010 BAF64826, 14B2 AAZ79294, Arg720 ACA96827, B4633–03 ABA34217, CAU 214 ABK62858, CP727 BAD24105, Dhaka12–03 ABA34219, Dhaka25–02 ABA34218, ISO1 AAP03062, ISO11 AAY85305, ISO21 AAZ17431, ISO27 AAZ17433, K12 BAD89095, Kor588 ACA96829, L26 ABV53272, Matlab13–03 ABA34220, MD844 BAF02906, MMC29 ACJ54792, MS310–0 BAG83242, N26–02 ABA34221, RU172 ABB17172, RV161–00 ABF67557, SK277 ACJ54800, T152 BAB88671, US6588 ACJ66743.

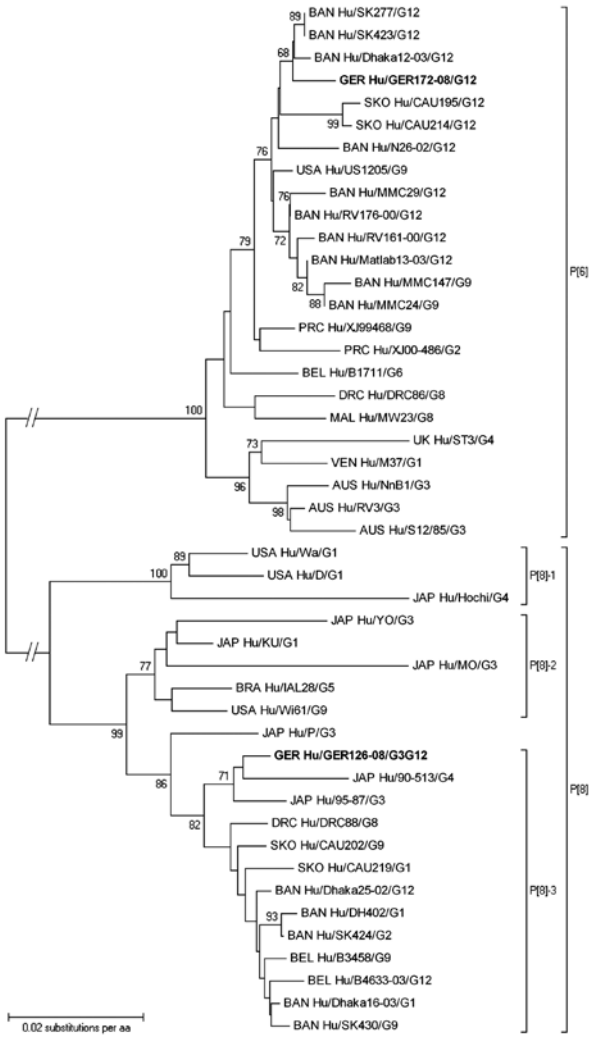

Figure 2. Phylogenetic dendrogram of viral protein 4 (VP4) P[6] and P[8] rotaviruses at the amino acid level. Bootstraps values (1,000 replicates) >65% are shown. The strain name is prefixed by the country of origin (AUS, Australia; BRA, Brazil; BAN, Bangladesh; BEL, Belgium; DRC, Democratic Republic of Congo; GER, Germany; JAP, Japan; MAL, Malawi; PRC, People's Republic of China; SKO, South Korea; UK, United Kingdom; USA, United States of America; VEN, Venezuela) as well as the viral host (Hu, human) and followed by the associated G genotype. **Boldface** indicates strains of this study. GenBank accession numbers of VP4 genes compared: P[6] B1711 ABU49763, CAU195 ABK62863, CAU214 ABK62864, Dhaka12–03 ABA34207, DRC86 AAY55972, M37 AAA57560, Matlab13–03 ABA34208, MMC147 ACJ54810, MMC24 ACJ54809, MMC29 ACJ54804, MW23 CAB92920, N26–02 ABA34209, NnB1 AAC68884, RV161–00 ABF67555, RV176–00 ABF67561, RV3 AAB05652, S12/85 AAC68883, SK277 ACJ54805, SK423 ACJ54803, ST3 ABV53292, US1205 AAC28852, XJ00–486 ABC49694, XJ99–468 ABC49698; P[8] 90–513 BAF80182, 95–87 BAA77555, B3458 ABV66093, B4633–03 ABA34205, CAU202 ABK62865, CAU219 ACD50869, D ABV53244, DH402 ACJ54815, Dhaka16–03 ABF50136, Dhaka25–02 ABA34206, DRC88 AAY55961, Hochi BAB32852, IAL28 ABV53260, KU BAE76023, MO BAA77543, P ABV53276, SK424 ACJ54811, SK430 ACJ54817, Wa AAA47290, Wi61 ABV53300, YO BAA77544.

were individually introduced into the local rotavirus diversity. Although GER172-08 is closely related to Southeast Asian strains, the origin of GER126-08 remains unclear. No conclusively related G12 sequence was published or detected in local piggeries.

A G3 VP7 sequence was found in addition in the stool sample GER126-08, however. Mixed infections are fairly common in crowded areas where population density is high and diverse rotavirus strains are co-circulating. They are required for reassortment, the major mechanism of rotavirus evolution (*13*,*14*). Pediatric wards match this setting during rotavirus seasons, because children with different rotavirus strains may be hospitalized simultaneously. Successive nosocomial rotavirus infections during hospitalization may facilitate asynchronous infections that favor reassortment (*15*). The case of the 10-year-old boy in this study fits into this pattern. Indications for recent reassortment events of strain GER126-08 have been detected consistently.

The detection of G12 rotavirus strains in Germany accentuates the need for extended multicenter studies to describe rotavirus diversity and control vaccine efficacy. Rotaviruses in animals should also be included to evaluate the origin of emerging genotypes.

Acknowledgments

We thank Bernd-Andreas Schwarz for providing the porcine rotavirus samples.

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References

1. Gentsch JR, Laird AR, Bielfelt B, Griffin DD, Bányai K, Ramachandran M, et al. Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. J Infect Dis. 2005;192:S1:S146–59.
2. Santos N, Hoshino Y. Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev Med Virol. 2005;15:29–56. DOI: 10.1002/rmv.448
3. Ruiz-Palacios GM, Pérez-Schael I, Velázquez FR, Abate H, Breuer T, Clemens SC, et al. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. N Engl J Med. 2006;354:11–22. DOI: 10.1056/NEJMoa052434
4. Vesikari T, Matson DO, Dennehy P, Van Damme P, Santosham M, Rodriguez Z, et al. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. N Engl J Med. 2006;354:23–33. DOI: 10.1056/NEJMoa052664
5. Rahman M, Matthijnssens J, Yang X, Delbeke T, Arijs I, Taniguchi K, et al. Evolutionary history and global spread of the emerging G12 human rotaviruses. J Virol. 2007;81:2382–90. DOI: 10.1128/JVI.01622-06

6. Gentsch JR, Glass RI, Woods P, Gouvea V, Gorziglia M, Flores J, et al. Identification of group rotavirus gene 4 types by polymerase chain reaction. J Clin Microbiol. 1992;30:1365–73.
7. Gouvea V, Glass RI, Woods P, Taniguchi K, Clark HF, Forrester B, et al. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J Clin Microbiol. 1990;28:276–82.
8. Matthijnssens J, Rahman M, Martella V, Xuelei Y, De Vos S, De Leener K, et al. Full genomic analysis of human rotavirus strain B4106 and lapine rotavirus strain 30/96 provides evidence for interspecies transmission. J Virol. 2006;80:3801–10. DOI: 10.1128/JVI.80.8.3801-3810.2006
9. Lambden PR, Cooke SJ, Caul EO, Clarke IN. Cloning of noncultivatable human rotavirus by single primer amplification. J Virol. 1992;66:1817–22.
10. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
11. Matthijnssens J, Ciarlet M, Rahman M, Attoui H, Bányai K, Estes MK, et al. Full genome-based classification of rotaviruses reveals a common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J Virol. 2008;82:3204–19. DOI: 10.1128/JVI.02257-07
12. Matthijnssens J, Ciarlet M, Rahman M, Attoui H, Bányai K, Estes MK, et al. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol. 2008;153:1621–9. DOI: 10.1007/s00705-008-0155-1
13. Patton JT, Vasquez-Del Carpio R, Tortorici MA, Taraporewala ZF. Coupling of rotavirus genome replication and capsid assembly. Adv Virus Res. 2007;69:167–201. DOI: 10.1016/S0065-3527(06)69004-0
14. Iturriza-Gómara M, Desselberger U, Gray J. Molecular epidemiology of rotaviruses: genetic mechanisms associated with diversity. In: Desselberger U, Gray J, editors. Gastroenteritis viruses. Amsterdam: Elsevier Science; 2003. p. 317–44.
15. Tauscher GI, Desselberger U. Viral determinants of rotavirus pathogenicity in pigs: production of reassortants by asynchronous coinfection. J Virol. 1997;71:853–7.

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Trends in US Hospital Admissions for Skin and Soft Tissue Infections

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Using data from the 2000–2004 US Healthcare Cost and Utilization Project National Inpatient Sample, we found that total hospital admissions for skin and soft tissue infections increased by 29% during 2000–2004; admissions for pneumonia were largely unchanged. These results are consistent with recent reported increases in community-associated methicillin-resistant *Staphylococcus aureus* infections.

During 1998–2004, *Staphylococcus aureus* was the most common cause of skin and soft tissue infections (SSTIs) in North America; frequency of these infections was 44.6%, and the rate of methicillin resistance among the isolates was 35.9% (*1*). Over the past decade, community-associated methicillin-resistant *S. aureus* (CA-MRSA) has become a notable public health problem; it accounts for 14% of invasive infections nationwide and 59% (range 15%–74%) of SSTIs among patients seeking treatment at emergency departments in 11 US cities (*2*,*3*). The emergence of CA-MRSA infections may have resulted in increased numbers of hospitalizations for SSTIs because of an increasing incidence of antimicrobial drug failure in outpatient treatment and more aggressive approaches to the management of these infections by physicians who are aware of the heightened risk of becoming infected with CA-MRSA. To determine whether hospital admissions for CA-MRSA are increasing, we analyzed data from the Healthcare Cost and Utilization Project National Inpatient Sample.

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DOI: 10.3201/eid1509.081228

The Study

This study was based on data from the Healthcare Cost and Utilization Project National Inpatient Sample (HCUP NIS) for the 5-year period 2000–2004. HCUP NIS is a stratified random sample from ≈20% of all US community hospitals. For 2004, the NIS contains all discharge data from >1,000 acute-care hospitals in 37 states, representing >8 million hospital stays.

We identified all hospital admissions in the HCUP NIS for which a principal diagnosis of SSTI was given. As defined by the Uniform Hospital Discharge Data Set, principal diagnosis is the condition "established after study to be chiefly responsible for occasioning the admission of the patient to the hospital for care" (http://wonder.cdc.gov).

SSTIs were defined as the following: 1) acute lymphadenitis (International Classification of Diseases, 9th Revision, Clinical Modification [ICD-9-CM] 683.XX); 2) carbuncle and furuncle (680.XX); 3) cellulitis and abscess of finger and toe (681.XX); 4) impetigo (684.XX); 5) infection (chronic) of amputation stump (997.62); 6) other cellulitis and abscess (682.XX); 7) other local infections of skin and subcutaneous tissue (686.XX); 8) abscess of anal and rectal regions (566); 9) chronic ulcer of other specified sites (707.8); 10) chronic ulcer of unspecified site (707.9); 11) decubitus ulcer (707); 12) infection due to other vascular device implant and graft (996.62); 13) pilonidal cyst with abscess (685); 14) postoperative wound infection (998.5X); 15) posttraumatic wound infection, not elsewhere classified (958.3); 16) ulcers of lower limbs, except decubitus (707.1X); 17) gangrene (785.4); and 18) necrotizing fasciitis (728.86).

To aid in interpretation of the data, we grouped the above-listed SSTIs into 3 mutually exclusive categories: 1) superficial infections predominantly caused by *S. aureus* or *Streptococcus pyogenes* (groups 1–7 above); 2); deeper or healthcare-associated infections more likely to involve anaerobic or gram-negative organisms (groups 8–16); and 3) infections typically associated with a high rate of mortality (groups 17–18). For each year of interest, all SSTI admissions were then stratified by type of infection and selected patient and hospital characteristics.

To provide a benchmark against which to interpret possible trends in SSTI-related hospital admissions, we generated similar series for all admissions with a principal diagnosis of infectious pneumonia, another common type of infection that frequently results in hospitalization but for which no trends were expected. Infectious pneumonia was defined to include 1) pneumococcal pneumonia (ICD-9-CM 481); 2) other bacterial pneumonia (482.XX)

Table 1. Estimated total number of US hospital admissions for SSTIs and infectious pneumonia, 2000–2004*

Principal diagnosis	2000	2001	2002	2003	2004	Change from 2000 to 2004
SSTI	674,939	701,672	757,858	810,768	869,777	194,838 (+28.9%)
Infectious pneumonia	1,202,387	1,177,972	1,229,204	1,272,686	1,172,304	–30,083 (–2.5%)

*SSTI, skin and soft tissue infection. Source: Healthcare Cost and Utilization Project National Inpatient Sample, 2000–2004.

Table 2. Number of hospital admissions with principal diagnosis of SSTI, by patient and infection characteristics and year*

Characteristics	Year 2000 (N = 674,939)	2001 (N = 701,672)	2002 (N = 757,858)	2003 (N = 801,768)	2004 (N = 869,777)
Age, y†					
<15	38,420 (5.7)	38,741 (5.5)	44,559 (5.9)	49,902 (6.2)	56,909 (6.5)
15–44	176,574 (26.2)	175,921 (25.1)	197,142 (26.0)	214,606 (26.5)	240,971 (27.7)
45–64	212,367 (31.5)	219,222 (31.2)	247,079 (32.6)	267,556 (33.0)	289,715 (33.3)
≥65	247,544 (36.7)	267,768 (38.2)	269,048 (35.5)	277,828 (34.3)	281,288 (32.3)
Sex†					
Male	341,040 (50.5)	347,784 (49.6)	381,763 (50.4)	406,525 (50.1)	443,325 (51.0)
Female	333,771 (49.5)	353,849 (50.4)	376,075 (49.6)	400,737 (49.4)	423,255 (48.7)
Race†					
White	364,634 (54.0)	368,901 (52.6)	372,013 (49.1)	409,037 (50.5)	439,295 (50.5)
Black	81,654 (12.1)	81,299 (11.6)	94,307 (12.4)	99,892 (12.3)	111,416 (12.8)
Hispanic/Latino	57,217 (8.5)	59,345 (8.5)	63,657 (8.4)	78,091 (9.6)	75,142 (8.6)
Asian or Pacific Islander	6,457 (1.0)	7,219 (1.0)	8,496 (1.1)	9,366 (1.2)	10,053 (1.2)
Native American	2,082 (0.3)	2,375 (0.3)	2,211 (0.3)	1,595 (0.2)	3,890 (0.5)
Other	11,873 (1.8)	11,233 (1.6)	16,304 (2.2)	14,135 (1.7)	13,937 (1.6)
Infection type (ICD-9-CM diagnosis code[s])					
Superficial infections	390,158 (57.8)	404,536 (57.7)	440,501 (58.1)	479,222 (59.1)	520,099 (59.8)
Acute lymphadenitis (683.XX)	4,570 (0.7)	4,341 (0.6)	4,230 (0.6)	4,324 (0.5)	4,291 (0.5)
Carbuncle and furuncle (680.XX)	1,001 (0.2)	1,182 (0.2)	1,349 (0.2)	1,900 (0.2)	2,298 (0.3)
Cellulitis and abscess of finger and toe (681.XX)	20,060 (3.0)	20,868 (3.0)	22,731 (3.0)	25,174 (3.1)	27,833 (3.2)
Impetigo (684.XX)	1,221 (0.2)	1,084 (0.2)	1,250 (0.2)	1,337 (0.2)	1,481 (0.2)
Infection (chronic) of amputation stump (997.62)	13,431 (2.0)	14,874 (2.1)	15,100 (2.0)	14,459 (1.8)	16,014 (1.8)
Other cellulitis and abscess (682.XX)	346,270 (51.3)	358,884 (51.2)	392,422 (51.8)	428,274 (52.8)	464,016 (53.4)
Other local infections of skin (686.XX)	3,605 (0.5)	3,302 (0.5)	3,419 (0.5)	3,755 (0.5)	4,167 (0.5)
Deeper and/or healthcare-associated infections	274,549 (40.7)	287,736 (41.0)	307,300 (40.6)	320,580 (39.5)	339,337 (39.0)
Abscess of anal and rectal region (566)	20,511 (3.0)	20,273 (2.9)	22,772 (3.0)	23,892 (3.0)	24,655 (2.8)
Chronic ulcer of other specified sites (707.8)	132 (0.0)	75 (0.0)	73 (0.0)	68 (0.0)	62 (0.0)
Chronic ulcer of unspecified site (707.9)	2,136 (0.3)	2,191 (0.3)	2,389 (0.3)	2,666 (0.3)	2,465 (0.3)
Decubitis ulcer (707.0)	37,116 (5.5)	38,637 (5.5)	36,816 (4.9)	38,167 (4.7)	39,706 (4.6)
Infection due to vascular device (996.62)	64,262 (9.5)	73,051 (10.4)	78,289 (10.3)	81,970 (10.1)	91,804 (10.6)
Pilonidal cyst with abscess (685.0)	2,262 (0.3)	2,167 (0.3)	2,447 (0.3)	2,496 (0.3)	2,604 (0.3)
Postoperative wound infection (998.5X)	115,694 (17.1)	120,344 (17.2)	134,783 (17.8)	138,393 (17.1)	144,768 (16.6)
Posttraumatic wound infection, NEC (958.3)	2,748 (0.4)	2,239 (0.3)	1,664 (0.2)	2,326 (0.3)	2,843 (0.3)
Ulcers of lower limbs, except decubitis (707.1X)	29,688 (4.4)	28,759 (4.1)	28,067 (3.7)	30,602 (3.8)	30,431 (3.5)
Often fatal infections	10,232 (1.5)	9,400 (1.3)	10,057 (1.3)	10,965 (1.4)	10,341 (1.2)
Gangrene (785.4)	5,628 (0.8)	4,814 (0.7)	5,155 (0.7)	5,546 (0.7)	4,331 (0.5)
Necrotizing fasciitis (728.86)	4,604 (0.7)	4,586 (0.7)	4,902 (0.7)	5,419 (0.7)	6,010 (0.7)
Hospital region					
Northeast	142,316 (21.1)	155,948 (22.2)	158,649 (20.9)	174,481 (21.5)	171,399 (19.7)
Midwest	152,132 (22.5)	159,442 (22.7)	173,353 (22.9)	175,144 (21.6)	193,602 (22.3)
South	253,388 (37.5)	269,323 (38.4)	297,122 (39.2)	316,028 (39.0)	351,721 (40.4)
West	127,102 (18.8)	116,958 (16.7)	128,734 (17.0)	145,115 (17.9)	153,056 (17.6)
Hospital location†					
Urban	570,554 (84.5)	592,908 (84.5)	645,261 (85.1)	691,795 (85.3)	755,095 (86.8)
Rural	103,664 (15.4)	108,763 (15.5)	112,597 (14.9)	118,563 (14.6)	114,682 (13.2)

*SSTI, skin and soft tissue infection; ICD-9-CM, International Classification of Diseases, 9th Revision, Clinical Modification; NEC, not elsewhere classified. Numbers are given as no. (%) admissions; N values for each year are provided. Source: Healthcare Cost and Utilization Project National Inpatient Sample, 2000–2004.
†Percentages may not add up to 100% due to missing data.

3); pneumonia caused by other specified organism (483.X) 4); bronchopneumonia, organism unspecified (485); and 5) pneumonia, organism unspecified (486).

Analyses were directed at discerning possible trends in SSTI-related hospital admissions at US acute-care hospitals over the 5-year period of study. Total SSTI admissions were estimated for each year by type of infection (i.e., superficial, deeper or healthcare-associated, often-fatal). Results also were stratified according to patient (e.g., age, sex) and hospital characteristics (e.g., urban vs. rural) available in the HCUP NIS. For comparison, we also examined total hospital admissions for infectious pneumonia.

Because the HCUP NIS undergoes periodic changes in its sampling frame and weighting method, as well as in data elements and definitions, we used the NIS Trends Supplemental (NIS-Trends) files to ensure comparability of data across the years of interest. The NIS-Trends files contain sampling weights and data elements that are consistently defined across all years of interest.

Analyses were primarily descriptive (i.e., no a priori hypotheses were made, and our results were not subjected to formal significance testing). All analyses were conducted by using the SAS Proprietary Software, Release 9.1 (SAS Institute, Cary, NC, USA).

The estimated total number of annual SSTI admissions to US acute-care hospitals rose steadily over the 5-year period, from 675,000 in 2000 to 869,800 in 2004, an increase of 194,000 (29%) admissions. In contrast, total admissions for infectious pneumonia fluctuated from year to year and were largely unchanged over this period (Table 1).

The increase in SSTI admissions was greatest among younger (age <65 years) rather than older patients (age 65–100 years) (37% vs. 14%, respectively) and for urban rather than rural hospitals (32% vs. 11%) (Table 2). The increase in SSTI admissions also was greatest among patients with superficial infections (e.g., cellulitis, abscess) rather than deeper or healthcare-associated infections (e.g., postoperative wound infection, infection due to vascular device) (33% vs. 24%).

Conclusions

Until recently, CA-MRSA was relatively uncommon. In 2000, for example, these isolates accounted for only 3% of staphylococcal isolates submitted to Minnesota laboratories (*4*). In recent years, however, CA-MRSA has become a major cause of SSTIs. The prevalence of MRSA among patients with SSTIs who sought treatment at 1 Los Angeles area emergency department increased from 29% in 2001 to 64% in 2004 (*5*).

While the estimated total number of SSTI admissions to US acute-care hospitals increased by ≈29% during 2000–2004, admissions for infectious pneumonia were largely unchanged. We are unaware of any other data to which our findings might be directly compared. Our results appear to be consistent, however, with those of previous studies that have reported an increasing prevalence of illness attributable to *S. aureus* infections in general and to MRSA in particular (*6*,*7*). Although we could not establish in our study whether the increase in the number of SSTI hospital admissions was a result of the growing prevalence of CA-MRSA (the HCUP-NIS does not report microbiologic data), we suspect that the 2 phenomena are closely linked—especially in light of the absence of any similar increase in hospital admissions for pneumonia, the most common community-associated infection requiring hospitalization. We therefore believe that the clinical and economic effects of CA-MRSA SSTIs are substantial and growing, and that this increase should be a focus of additional research.

Funding for this research was provided by Astellas Pharma US, Inc. Although the study sponsor participated in discussions concerning the design and conduct of the study, manuscript preparation, and the decision to submit the manuscript for publication, all final decisions were made by the authors.

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References

1. Moet GJ, Jones RN, Biedenbach DJ, Stilwell MG, Fritsche TR. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: report from the SENTRY Antimicrobial Surveillance Program (1998–2004). Diagn Microbiol Infect Dis. 2007;57:7–13. DOI: 10.1016/j.diagmicrobio.2006.05.009
2. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA. 2007;298:1763–71. DOI: 10.1001/jama.298.15.1763
3. Moran GJ, Krishnadasan A, Gorwitz RJ, Fosheim GE, McDougal LK, Carey RB, et al. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med. 2006;355:666–74. DOI: 10.1056/NEJMoa055356
4. Naimi TS, LeDell KH, Boxrud DJ, Groom AV, Steward CD, Johnson SK, et al. Epidemiology and clonality of community-acquired methicillin-resistant *Staphylococcus aureus* in Minnesota, 1996–1998. Clin Infect Dis. 2001;33:990–6. DOI: 10.1086/322693
5. Moran GJ, Amii RN, Abrahamian FM, Talan DA. Methicillin-resistant *Staphylococcus aureus* in community-acquired skin infections. Emerg Infect Dis. 2005;11:928–30.
6. Noskin GA, Rubin RJ, Schentag JJ, Kluytmans J, Hedblom EC, Jacobson C, et al. National trends in *Staphylococcus aureus* infection rates: impact on economic burden and mortality over a 6-year period. Clin Infect Dis. 2007;45:1132–40. DOI: 10.1086/522186
7. Klein E, Smith DL, Laxminarayan R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999–2005. Emerg Infect Dis. 2007;13:1840–6.

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Who is this man?

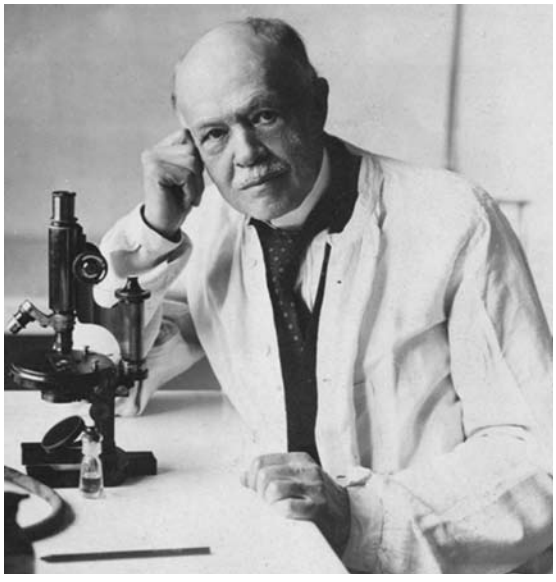

Image courtesy of David M. Morens

Here is a clue: he discovered that epidemic typhus is transmitted by lice.

Who is he?

A) Charles-Jules-Henri Nicolle

B) Stanislaus Joseph Matthias von Prowazek

C) Howard Taylor Ricketts

D) Henrique da Rocha Lima

E) Hans Zinsser

Decide first. Then turn the page.

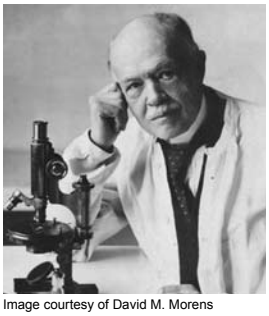

Image courtesy of David M. Morens

Charles-Jules-Henri Nicolle

Myron G. Schultz and David M. Morens

Shown in this photograph is Charles-Jules-Henri Nicolle (1866–1936), a physician, microbiologist, novelist, philosopher, and historian. From 1903 until his death in 1936, he was Director of the Institut Pasteur in Tunis, Tunisia. Nicolle's many accomplishments include the discovery that epidemic typhus is transmitted by body lice (*Pediculus humanis corporis*), discovery of the phenomenon of inapparent infection, and possibly the first isolation of human influenza virus after experimental transmission. Nicolle made many other fundamental contributions to knowledge of infectious diseases. This year is the centenary of his discovery about typhus transmission, made in the summer of 1909, for which he was awarded the 1928 Nobel Prize in Physiology or Medicine.

Nicolle was born on September 21, 1866, in Rouen, the ancient capital of Normandy, France. He obtained a classical education and was greatly attracted to literature, history, and the arts, interests he nurtured throughout his life. Bowing to the wish of his physician father, however, Nicolle studied medicine. After 3 years at the medical school in Rouen, he proceeded to Paris for further training and received a medical degree from the Institut Pasteur in 1893. At 27 years of age, Nicolle returned to his hometown, where he served as a member of the medical faculty and as Director of the Bacteriological Laboratory at L'École préparatoire de médecine et pharmacie de Rouen. His 8 years in Rouen were difficult: his position was untenured, his colleagues were reluctant to accept his modern ideas about bacteriology, and he experienced a hearing loss that prevented him from effectively using a stethoscope. These challenges may have motivated him to take a leap that he might otherwise not have taken when the post of directorship of the Institut Pasteur in Tunis became open. It was offered to his elder brother, Maurice (1862–1932), an established experimental scientist, who refused it. Charles then applied and obtained the position.

Nicolle arrived in Tunis in 1902, when he was 36 years old. North Africa was a good place to study infectious diseases, including brucellosis, diphtheria, leishmaniasis, leprosy, malaria, measles, Mediterranean spotted fever, relapsing fever, scarlet fever, tuberculosis, and typhus. Of all the problems Nicolle faced in Tunis, however, epidemic typhus was, in his words, "the most important and the least explored." He studied it for the next 7 years. He was well aware of the clinical presentation of typhus—its triad of fever, rash, and stupor—and of its link to poverty. Throughout history, typhus had been a highly communicable and frequently fatal disease. Before it began to be understood as a single infectious disease distinguished epidemiologically from typhoid (in the mid to late 19th century), typhus had been considered a collection of distinctive diseases that affected specific populations. It devastated armies during wars ("war typhus") and prisoners living under unsanitary conditions ("jail typhus" or "jail fever"); it affected displaced populations suffering from famine, floods, and other natural disasters; and in general, it was a disease of poverty.

In Tunis, typhus struck in seasonal waves during the cooler months and disappeared during the summer. It spread through overcrowded prisons, asylums, and tent villages, taking a heavy toll in hospitals among admissions personnel and sometimes even among examining physicians. Most of the doctors in the Tunisian health system, especially those in rural districts, had contracted typhus; approximately one third of them died from it. Nicolle's first encounter with typhus could have potentially been his last. In 1903, he escaped death when at the last moment he cancelled a trip to investigate a prison outbreak. His 2 colleagues went on to the prison without him and spent the night there; both became ill with typhus and died.

Nicolle's discovery of how typhus is transmitted came from observations at the entrance and waiting room of the Sadiki Hospital, which primarily served indigent patients.

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DOI: 10.3201/eid1509.090891

He often had to step over the bodies of typhus-infected patients who had fallen and died at the doorway. Nicolle observed that typhus patients who were admitted spread their infections to others up to the point at which they entered the hospital waiting room. Included among these secondary cases were persons who took charge of their clothing. However, patients became completely noninfectious as soon as they were bathed and dressed in a hospital uniform. They could then enter the general wards without posing a risk to others. Once Nicolle realized this, he reasoned that lice on patients' clothes were most likely the vectors.

To test his hypothesis about lice, Nicolle requested and promptly received a chimpanzee (*Pan troglodytes*) from his mentor, Pierre-Paul-Émile Roux (1853–1933), at the Paris Institut Pasteur. Nicolle injected the chimpanzee with blood from a typhus patient. Twenty-four hours later, the chimpanzee was febrile, had new skin eruptions, and was prostrate. Because chimpanzees were costly, Nicolle then injected a toque macaque (*Macaca sinica*) with blood from the ill chimpanzee. Thirteen days later the macaque became febrile. Nicolle fed 29 lice on the ill macaque, and over the next few days transferred the lice to feed on other macaques. Eventually, macaques in this latter group became ill as well.

Thus, in June 1909, Nicolle reproduced typhus in a chimpanzee; in August 1909, he demonstrated that lice are the carriers of typhus; and in September 1909, he communicated his discovery to the French Académie des sciences. In these simple experiments, Charles Nicolle had solved the mystery surrounding the transmission of one of humankind's most dreaded scourges, a disease that had been a major force in shaping world history. Later research showed that the principal transmission method was not the bites of lice but the excrement of lice rubbed into the skin or eyes.

Hans Zinsser (1878–1940), an American microbiologist and historian, dedicated his classical work, Rats, Lice and History, to Charles Nicolle "with affectionate friendship." In his autobiography, Zinsser speaks of Nicolle's qualities as a scientist:

> Nicolle was one of those men who achieve their success by long preliminary thought, before an experiment is formulated, rather than by the frantic and often ill-conceived experimental activities that keep lesser men in ant-like agitation... Nicolle did relatively few and simple experiments. But every time he did one, it was the result of long hours of intellectual incubation during which all possible variants had been considered and were allowed for in the final tests. Then he went to the point, without wasted motion … In the case of the louse discovery, Nicolle had carried out no more than a half-dozen decisive experiments after years of observation of the disease and its epidemiology. In this instance, his experiments were easily confirmed.

Indeed, in the year after Nicolle's typhus discovery, Howard Taylor Ricketts (1871–1910) and Russell Morse Wilder (1885–1959), working in Mexico, confirmed louse transmission of typhus. In 1916, Henrique da Rocha-Lima (1879–1956) identified the causative organism and named it *Rickettsia prowazeki* in memory of Ricketts and Stanislaus Joseph Matthias von Prowazek (1875–1915), both of whom had died of typhus contracted during their scientific investigations.

Although Nicolle is not credited with discovering the cause of human influenza, his contributions were seminal. In 1903, when he had just joined the Institut Pasteur in Tunisia, his mentor Émile Roux reviewed the literature on "filter-passing" agents (hypothetical subbacterial agents that passed through Berkfeld and Chamberland filters). Roux identified 10 of them that he believed to be scientifically proven as causative agents of disease, among them what we now know to be viruses and mycoplasmas. Working at Turkey's Imperial Institute of Bacteriology, Nicolle's brother Maurice and colleagues had isolated the filter-passing agent of rinderpest (later characterized as a paramyxovirus). Charles Nicolle, who had also worked with rinderpest, was familiar with these new techniques.

When the deadly influenza pandemic struck in 1918, Nicolle was among the few scientists in the world prepared to study its etiology. At the time, the cause of influenza was unknown, but many doubted the conventional explanation that it was a bacterial disease. Beginning on September 1, 1918, Nicolle injected Chamberland-filtered and unfiltered sputum samples from ill patients into human volunteers and into monkeys, reproducing in some experiments a febrile influenza-like illness. However, the scarcity of clinical material and the rapidity with which the epidemic advanced precluded large-scale controlled studies. Within a few months, a Japanese group appeared to reproduce and extend the results of the 2 French scientists, but other investigators had trouble doing so. As the pandemic faded into endemicity, further experimentation became difficult for all researchers. When influenza viruses were eventually isolated and characterized in mice and in ferrets more than a decade later, Nicolle was finally acknowledged as having made the first isolation and as having taken the first important steps toward finding influenza's cause.

In addition to increasing knowledge about typhus and influenza, Nicolle made important contributions to the un-

derstanding of brucellosis, leishmaniasis, measles, rinderpest, scarlet fever, Mediterranean spotted fever, toxoplasmosis, trachoma, and tuberculosis. Perhaps his greatest discovery, a critical key to understanding the epidemiology of many infectious diseases, was characterization of the phenomenon of inapparent infection, the acquisition and transmission of infection without signs of illness. This line of work began with Nicolle's observations on experimental typhus. He learned that guinea pigs were good hosts for the typhus organism and showed that certain guinea pigs could have apyretic typhus after a primary infection of pyretic typhus. Nicolle extended his observation to other infections—viral, bacterial, and parasitic—finding similar phenomena in each. As Charles-Edward Amory Winslow (1877–1957) emphasized in his classical work, The Conquest of Epidemic Diseases: A Chapter in the History of Ideas (1943), inapparent infection is one of the most important concepts in infectious disease epidemiology, and it had for centuries been one of the key missing links, which prevented full understanding of the principles of disease transmission. Inapparent infection of symptomless carriers is now generally accepted as the source for dissemination of many communicable diseases. Nicolle considered it his most important discovery.

Nicolle wrote several philosophical works, including Destin des maladies infectieuses (1933); La nature; conception et morale biologiques (1934); Responsabilités de la médecine (1936); and La destinée humaine (1941). Nicolle also wrote fanciful stories, such as Le pâtissier de Bellone (1913), Les deux larrons (1929), and Les contes de Marmouse et de ses hôtes (1930). His novels brought him great pleasure and a circle of admiring readers.

Nicolle's discovery of the means of transmission of typhus can be viewed as both a beginning and an end. It ended a 20-year epoch in which arthropods were found to be the vectors of major diseases of animals and humans, including not only typhus but also African trypanosomiasis, American trypanosomiasis, dengue, filariasis, malaria, relapsing fever, Texas cattle fever, and yellow fever. This epoch was as successful in the history of medicine as had been the phenomenal development of bacteriology in the decades immediately preceding it. Nicolle's discovery was also the beginning of the end of epidemic typhus. In the late 1930s, Paul Müller (1899–1965) discovered that dichlorodiphenyltrichloroethane (DDT) was highly effective for killing lice and other insects. During World War II, several potentially severe epidemics of typhus, especially the epidemic in Naples, Italy, in 1943–1944, were averted by dusting at-risk populations with DDT. Epidemic typhus had reigned for centuries, extinguishing millions of lives prematurely. Now it is an uncommon epidemic disease. The combined discoveries of Nicolle and Müller are compelling proof of the melioristic notion that the world becomes a better place through sustained human effort. Charles Nicolle, a modest Renaissance man who toiled in Africa, far from the scientific limelight of Berlin, New York, and Paris, is remembered for forever changing biomedical science and for having contributed to saving the lives of millions.

Dr Schultz is a senior medical officer working in the Global Disease Detection Operations Center at the Centers for Disease Control and Prevention (CDC). Formerly, he was director, Parasitic Diseases Division, and worked in the Epidemiology Program Office at CDC. He has published articles on medical history in numerous medical journals and authors CDC's annual contest, Great Moments in Public Health.

Dr Morens is an epidemiologist and historian who serves as senior advisor to the director, National Institute of Allergy and Infectious Diseases, and as associate editor of Emerging Infectious Diseases. His interests include infectious disease pathogenesis, determinants of disease emergence, and the history of infectious diseases.

Suggested Reading

1. Gross L. How Charles Nicolle of the Pasteur Institute discovered that epidemic typhus is transmitted by lice: reminiscences from my years at the Pasteur Institute in Paris. Proc Natl Acad Sci U S A. 1996;93:10539–40. DOI: 10.1073/pnas.93.20.10539
2. Nicolle C, Comte C, Conseil L. Transmission expérimentale du typhus exanthématique par le pou du corps. Comptes Rendus Hebdo-Madaires des Séances de l'Académie des Sciences. 1909; 149:486–9.
3. Pelis K. Charles Nicolle, Pasteur's imperial missionary: typhus and Tunisia. Rochester (NY): University of Rochester Press; 2006.
4. Yoeli M. Charles Nicolle and the frontiers of medicine. N Engl J Med. 1967;276:670–5.
5. Zinsser H. Rats, lice and history. Boston: Little, Brown; 1935.
6. Zinsser H. As I remember him; the biography of R.S. Boston: Little, Brown; 1940.

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Genomic Diversity of Oseltamivir-Resistant Influenza Virus A (H1N1), Luxembourg, 2007–08

To the Editor: The prevalence of oseltamivir-resistant influenza viruses A (H1N1) (ORVs) increased dramatically worldwide during the winter of 2007–08 (*1*). Recent reports indicated that by early 2009 most influenza virus (H1N1) strains were resistant to oseltamivir (*2*). Resistant viruses were transmitted readily and were as viable and pathogenic as oseltamivir-sensitive viruses (OSVs) (*3*,*4*). The His275Tyr (N1 numbering) mutation in the neuraminidase (NA) genes of influenza virus A (H1N1) that confers resistance to oseltamivir has previously been associated with impaired virus replication, infectivity, and pathogenicity (*5*,*6*).

We investigated the genetic diversity in all 8 gene segments of representative ORVs and OSVs collected during December 2007–March 2008 by the National Influenza Sentinel Surveillance System in Luxembourg (www.lns.public.lu/statistiques/grippe). Phylogenetic analyses were performed by using MEGA version 4.0 (*7*). Tree topology and posterior probabilities were calculated by using MrBayes version 3 (*8*). The sequences have been submitted to GenBank (accession nos. FM174406–60, FN401430–45, and FN401487–FN401518).

Among 140 viruses, 34 strains (24.3%) had the oseltamivir-resistant genotype (Tyr275) in the NA gene. Bayesian analyses of NA genes showed that ORVs formed a distinct cluster supported by high posterior probability (1.00) on the common node (Figure). One resistant strain (LNS-365) was more closely related to OSVs (minimal Kimura distance 0.3%, 4 nt) than to ORVs (minimal Kimura distance 0.5%, 6 nt). In NA protein, 33 ORVs showed the common Asp354Gly substitution in addition to the Tyr275 mutation. The resistant outlier LNS-365 encoded Asp354 like all other OSVs (n = 106). Similarly, only 4 other resistant strains from Europe from the same season shared Asp354 with all 2007–08 sensitive influenza virus (H1N1) strains (n = 251) available in public databases.

A total of 18–44 selected sequences from each of the other genes of ORVs and OSVs were generated to investigate which other genetic markers cosegregated with the resistant genotype. Sequences derived from most of the other genes (polymerase proteins PB1 and PA, hemagglutinin, nucleoprotein, matrix protein, nonstructural protein) of ORVs and OSVs were phylogenetically interspersed with no distinct clustering. In contrast, matching the phylogeny of NA, PB2 sequences of genotypically resistant strains (n = 14) formed a distinct cluster supported by high posterior probabilities (1.00) and separate from all OSVs (n = 16) and the resistant outlier LNS-365 (Figure). On the PB2 amino acid level, all OSVs and the resistant outlier LNS-365 shared Pro453, whereas all ORV encoded serine at the same position (Ser453). The outlier LNS-365 differed only by 2 aa from OSVs but by 4 aa from the closest resistant strain.

All published PB2 sequences for influenza virus (H1N1) strains col-

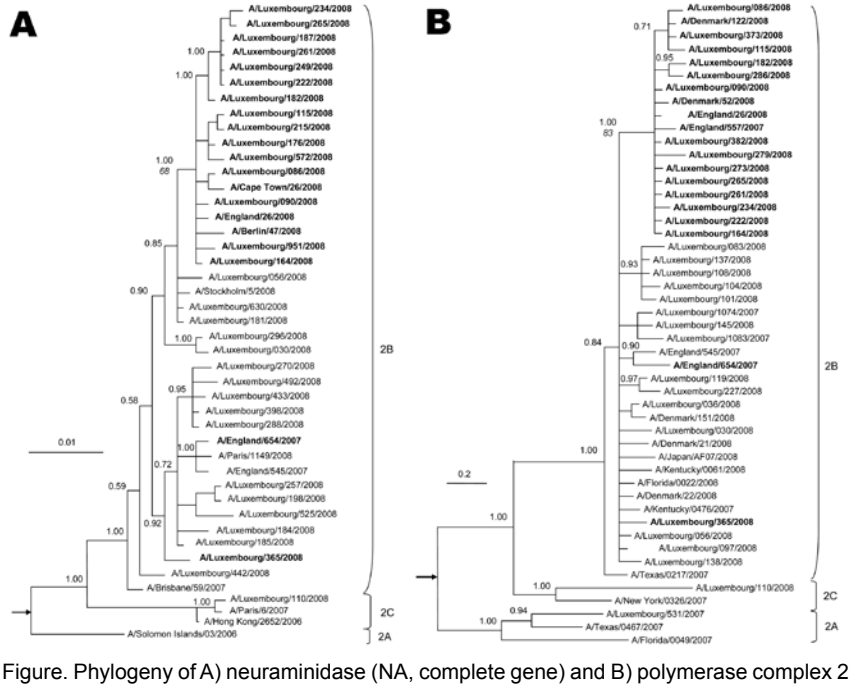

Figure. Phylogeny of A) neuraminidase (NA, complete gene) and B) polymerase complex 2 (C-terminal 1,300 nt) genes for selected influenza viruses A (H1N1) from Luxembourg and other countries. Subclades are identified to the right of each tree. The best-approximating model of nucleotide evolution was the general time reversible model with a gamma rate distribution and this model was used for the Bayesian analysis. Markov chain Monte Carlo sampling was implemented in MrBayes version 3 (*8*). In all cases, 6 chains with at least 4 million generations were calculated (10% burn-in removed). At least 2 independent runs of each analysis were performed. Posterior probabilities (indicated on important nodes) of the consensus tree topologies were estimated by sampling likelihood parameters every 125 generations. **Boldface** indicates sequences of oseltamivir-resistant influenza viruses A (H1N1) with the Tyr275 mutation in NA. In MEGA version 4, a neighbor-joining tree with 10,000 replicates was generated to calculate bootstrap values, shown in italics on the node dividing resistant and sensitive strains. Scale bars indicate nucleotide substitutions per site. The trees are rooted on A/New Caledonia/01/1999 and A/BrevigMission/1918 (indicated by arrows).

lected since 1918 (n = 720) encoded either Pro453 or His453. Until the emergence of ORVs in 2007, Ser453 was only present in 3 other strains (A/Wilson-Smith/1933 and 2 strains from 1976 and 1988). Located on the surface of the PB2 cap-binding domain (*9*), the Pro453Ser mutation may influence polymerase function and virus replication. The fact that PB2 sequences of ORVs and OSVs are phylogenetically segregated suggests a link between the genetic background and the unexpected fitness of ORVs. There was no amino acid mutation in any of the other genes that segregated in the same way between ORVs and OSVs other than Ser453 (PB2).

Only 1 OSV strain from Luxembourg in 2007–08 (LNS-110) was derived from subclade 2C, unlike the other 139 influenza virus (H1N1) strains (subclade 2B, Figure). Like many other subclade 2C strains, which were recently identified, this virus encoded the amantadine-resistance marker Asn31 in the matrix 2 protein (*10*). Although we did not identify any reassortments between ORVs and OSVs, double-resistant strains may result from cocirculation of amantadine-resistant and ORVs in the same region.

The phylogeny of ORVs identified worldwide (*2*) indicates multiclonal emergence of resistance, which suggests that OSVs may contain low levels of ORV subpopulations. Using pyrosequencing, we determined the incidence and level of mixed alleles in codon 275 of the NA gene (CAT, sensitive and TAT, resistant). In 98 clinical specimens (78 sensitive and 20 resistant strains) no minority alleles were reliably detected above the 3% threshold of the assay. Six OSVs with values between 2.1% and 2.9% were further analyzed by cloning of partial NA genes. No evidence of ORVs was found (Tyr275) in 227 clones.

In summary, we have described amino acid markers in NA (Gly354) and PB2 (Ser453) proteins, which were present in ORVs but absent in all OSVs from Luxembourg in 2007–08. ORVs without this background did not spread as efficiently and were rarely found in Europe. At least 1 resistant virus was more similar to OSV, which suggests ≥2 clones of resistant viruses in Luxembourg, potentially with different viral fitness. We speculate that the unexpected fitness of the 2007–08 influenza viruses (H1N1) may be caused by a new genetic background that is most likely encoded in the PB2 gene.

Acknowledgments

We thank Emilie Charpentier, Aurélie Sausy, and Sébastien de Landtsheer for technical assistance, and the clinicians who participated in the Influenza Sentinel Surveillance in Luxembourg.

N.A.G. was supported by an Aides à la Formation-Recherche fellowship of the Ministry of Research and Higher Education, Luxembourg.

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DOI: 10.3201/eid1509.090452

References

1. Recommended composition of influenza virus vaccines for use in the 2009 southern hemisphere influenza season. Wkly Epidemiol Rec. 2008;83:366–72.
2. World Health Organization. Influenza A(H1N1) virus resistance to oseltamivir—2008/2009 influenza season, northern hemisphere [cited 2009 Mar 25]. Available from http://www.who.int/csr/disease/influenza/H1N1webupdate20090318 ed_ns.pdf
3. Meijer A, Lackenby A, Hungnes O, Lina B, van der Werf S, Schweiger B, et al. Oseltamivir-resistant influenza A (H1N1) virus, Europe, 2007–08 season. Emerg Infect Dis. 2009;15:552–60. DOI: 10.3201/eid1504.081280
4. Rameix-Welti MA, Enouf V, Cuvelier F, Jeannin P, van der Werf S. Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir. PLoS Pathog. 2008;4:e1000103. DOI: 10.1371/journal.ppat.1000103
5. McKimm-Breschkin J, Trivedi T, Hampson A, Hay A, Klimov A, Tashiro M, et al. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. Antimicrob Agents Chemother. 2003;47:2264–72. DOI: 10.1128/AAC.47.7.2264-2272.2003
6. Yen HL, Herlocher LM, Hoffmann E, Matrosovich MN, Monto AS, Webster RG, et al. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. Antimicrob Agents Chemother. 2005;49:4075–84. DOI: 10.1128/AAC.49.10.4075-4084.2005
7. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
8. Ronquist F, Huelsenbeck JP. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572–4. DOI: 10.1093/bioinformatics/btg180
9. Guilligay D, Tarendeau F, Resa-Infante P, Coloma R, Crepin T, Sehr P, et al. The structural basis for cap binding by influenza virus polymerase subunit PB2. Nat Struct Mol Biol. 2008;15:500–6. DOI: 10.1038/nsmb.1421
10. Niman H. Emergence and fixing of antiviral resistance in influenza A via recombination and hitch hiking. 2009 Feb 10 [cited 2009 Mar 25]. Available from http://hdl.handle.net/10101/npre.2009.2832.1

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Tokyo-172 BCG Vaccination Complications, Taiwan

To the Editor: BCG (*Mycobacterium bovis* BCG) is a vaccine for preventing childhood tuberculosis (TB), especially military and meningeal TB. Because Taiwan has an annual TB incidence rate of ≈70 cases/100,000 persons, the National Immunization Program has included neonatal BCG vaccination since 1965. The coverage rate has remained at 97% since 2001. According to the Taiwan Tuberculosis Registry, the median rate of TB infections diagnosed in patients <10 years of age during 2005–2007 was 0.39% (60 cases) (*1*). The risk of developing childhood extrapulmonary TB without lung involvement is highest among children <5 years of age.

In 1965, the World Health Organization registered freeze-dried Tokyo-172 seed lot as an international reference vaccine strain (*2*). Tokyo-172 BCG is currently used in Taiwan, Japan, and South Korea. The vaccine is recommended as less reactogenic. Because intradermal injection is recognized as a more effective BCG administration route (*3*), it is practiced in Taiwan, while a multiple puncture method is used in Japan and South Korea. In addition, 10% BCG (Danish strain) vaccinations for infants are administrated intracutaneously in South Korea (*4*). Although BCG is effective in preventing progressive primary TB, adverse reactions to the vaccine do occur. A systemic review of adverse reactions has been established in Japan (*5*) but not in South Korea (*4*) and Taiwan (*6*).

During 1951–2004, a total of 39 cases of severe adverse vaccine reactions were reported in Japan, with an incidence rate of 0.182 cases of reactions/million vaccinations. Of the 39 cases, 27 patients (69.2%) had bone and joint involvement, and 13 (33.3%) had primary immunodeficiency (*4*). One patient had both complications. The BCG vaccine was initially produced in Taiwan using Pasteur-1173 P2 strain (0.025 mg/0.1mL) and changed to the less reactogenic Tokyo-172 strain (0.05 mg/0.1 mL) in 1979. From 1998 through 2007, 14 patients applied for compensation through the vaccine injury compensation program for BCG-caused adverse reaction, and 6 claims were confirmed. Of the 6 confirmed BCG complications cases, 5 patients had humeral or sternal osteomyelitis and 1 patient died from a disseminated BCG infection. Accordingly, in 2002–2006 the risk for BCG osteitis/osteomyelitis and disseminated BCG infection was 3.68 and 0.9 per million, respectively (*6*). Incidence of severe complications was higher than that documented in Japan.

Because Taiwan lacks diagnosis and postmarketing surveillance system, BCG-related complications might be underreported. As part of initiating a comprehensive adverse events surveillance, a laboratory program to differentiate *M. bovis* BCG from other species of the *M. tuberculosis* complex was established to monitor local adverse events (injection-site abscess, lymphadenitis) and severe complications (suppurative lymphadentitis, BCG osteitis/osteomyelitis, and disseminated BCG infection) among vaccinated children.

During 2005–2007, 19 clinical specimens (6 biopsy samples and 13 bacterial isolates) of suspected BCG-infection childhood TB cases were sent to the Taiwan Centers for Disease Control. To differentiate among *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG, DNA samples were initially screened using a GenoType kit (Hain Lifescience GmbH, Nehren, Germany), multiplex PCR (*7*), and pncA sequencing (*8*). In addition, spoligotyping was performed with a commercial kit (Isogen Bioscience BV, Maarssen, the Netherlands). An additional multiplex PCR (*9*) was used to differentiate vaccine strains. Medical charts were reviewed to determine sites of involvement and severity of complications.

Of the 19 patients, 1 (5.3%), 2 (10.5%), 15 (78.9%), and 1 (5.3%) were infected with *M. tuberculosis* complex, *M. tuberculosis*, *M. bovis* BCG, and *M. abscessus*, respectively. All identified *M. bovis* BCG isolates had the same spoligotype as the Tokyo-172 vaccine strain. The median age of BCG-related complication patients was 2 years (range 1–9 years), and the male:female ratio was 1.5. Of the 15 *M. bovis* BCG–infected patients, 14 had extrapulmonary sites of involvement, including 8 bone and joint, 3 suppurative lymphadenitis in axillary lymph node, 2 subcutaneous abscess away from the injection site, 1 injection-site abscess, and 1 disseminated BCG infection (Table).

According to an international survey, the estimated rate of osteitis/osteomyelitis is 1–700/1,000,000 vaccinated newborns or infants with different strain-derived BCG (*10*). In Taiwan, the estimated incidence of BCG osteitis/osteomyelitis was 12.9 cases (8/621,853; 95% confidence interval 4–21.8) per million vaccinations during 2005–2007. Previously reported complications of BCG osteitis/osteomyelitis related to Tokyo-172 strain might be underestimated. The association between administration route and osteitis/osteomyelitis with Tokyo-172 BCG remains obscure. The potency and safety of BCG prepared from Tokyo-172 stain are under reevaluation as one of the action plans of National TB Program. The stability and quality control of vaccines strain, production processes, and intradermal injection techniques are being reappraised.

Furthermore, a policy of enhanced childhood TB surveillance was implemented in 2007, and clinicians were advised to send clinical specimens to the Centers for Disease Control in Taiwan for differential diagnosis of *M. bovis* BCG for patients <5 years of

Table. Characteristics *Mycobacterium bovis* BCG complication cases, Taiwan, 2005–2007*

Patient no.	Sex/age at diagnosis, y	Year reported	Specimen	Diagnosis and site of involvement
1	F/2	2005	Biopsy sample	BCG osteitis/osteomyelitis, right ankle
2	M/1	2005	Bacterial isolate	Subcutaneous abscess, left anterior chest wall
3	M/2	2005	Bacterial isolate	Severe combined immunodeficiency, disseminated BCGitis
4	M/9	2005	Bacterial isolate	Suppurative lymphadenitis
5	F/1	2005	Bacterial isolate	Injection-site abscess
6	M/1	2005	Biopsy sample	Suppurative lymphadenitis
7	M/2	2006	Bacterial isolate	BCG osteitis/osteomyelitis, right distal femoris
8	M/2	2006	Bacterial isolate	BCG osteitis/osteomyelitis
9	F/1	2006	Bacterial isolate	BCG osteitis/osteomyelitis, left distal femoris
10	F/1	2006	Bacterial isolate	BCG osteitis/osteomyelitis, left distal radius
11	F/2	2007	Bacterial isolate	BCG osteitis/osteomyelitis, right knee
12	M/1	2007	Bacterial isolate	Subcutaneous abscess, left wrist
13	M/2	2007	Biopsy sample	BCG osteitis/osteomyelitis, right ankle
14	F/1	2007	Bacterial isolate	Suppurative lymphadentitis
15	M/2	2007	Bacterial isolate	BCG osteitis/osteomyelitis, left proximal tibia

*BCGitis, disseminated BCG infection.

age. In particular, suspected childhood TB patients without an identifiable TB contact and with normal immune status were subjected to further investigations. Multidisciplinary management, including enhanced laboratory diagnosis of atypical bony lesions in infants and children, is recommended for any suspected TB infection. Once BCG-related infection is confirmed, medical treatment has to be consistent.

Acknowledgments

We thank Steve H. S. Kuo, Toru Mori, and Jen Suo for comments and Chen-Che Chiu and Chien-Chung Huang for excellent technical assistance.

This study was supported by grant DOH97-DC-2501 from Taiwan Centers for Disease Control, Department of Health.

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DOI: 10.3201/eid1509.081336

References

1. Taiwan Centers for Disease Control. Statistics of communicable diseases and surveillance report, tuberculosis, 2005–2007. Taipei, Taiwan: Taiwan Centers for Disease Control.
2. Yamamoto S, Yamamoto T. Historical review of BCG vaccine in Japan. Jpn J Infect Dis. 2007;60:331–6.
3. Plotkin SA, Orenstein WA, Offit PA. Vaccines, 5th ed. Philadelphia: Saunders Elsevier; 2008:867.
4. Kim SH, Kim SY, Eun BW, Yoo WJ, Park KU, Choi EH, et al. BCG ostemyelitis caused by the BCG Tokyo strain and confirmed by molecular method. Vaccine. 2008;26:4379–81.
5. Toida I, Nakata S. Severe adverse reaction with Japanese BCG vaccine: a review. Kekkaku. 2007;82:809–24.
6. Sheu GC, Yang SL, Lee CD, Liu DP. Adverse events induced by BCG immunization in Taiwan. Taiwan Epidemiology Bulletin. 2008;24:357–71.
7. Yeboah-Manu D, Yates MD, Wilson SM. Application of a simple multiplex PCR to aid in routine work of the mycobacterium reference laboratory. J Clin Microbiol. 2001;39:4166–8. DOI: 10.1128/JCM.39.11.4166-4168.2001
8. Scorpio A, Collins D, Whipple D, Cave D, Bates J, Zhang Y. Rapid differentiation of bovine and human tubercle bacilli based on a characteristic mutation in the bovine pyrazinamidase gene. J Clin Microbiol. 1997;35:106–10.
9. Bedwell J, Kairo SK, Behr MA, Bygraves JA. Identification of substrains of BCG vaccine using multiplex PCR. Vaccine. 2001;19:2146–51. DOI: 10.1016/S0264-410X(00)00369-8
10. World Health Organization. Supplementary information on vaccine safety by World Health Organization: Part 2: Background and rates of adverse events following immunization. Geneva: The Organization; 2000.

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Reemergence of Bolivian Hemorrhagic Fever, 2007–2008

To the Editor: Bolivian hemorrhagic fever (BHF) was first described in 1959 during outbreaks affecting isolated human communities in eastern Bolivia. However, it was not until 1963 that the etiologic agent, Machupo virus, was isolated from the spleen of a patient who died from this disease (*1*). Although no cases were reported between 1976 and 1993, an outbreak occurred in 1994 and sporadic cases have been observed since then.

In February and March 2007, at least 20 suspected BHF cases (3 fatal) were reported to the El Servicio Departamental de Salud (SEDES) in Beni,

Bolivia. In February 2007, physicians at the Hospital Santa Maria Magdalena reported 3 male patients (23, 27, and 29 years of age), who worked at a ranch in Magdalena, Itenez Province (13°14′0″S, 64°12′0″W). The patients sought treatment for fever, gingivorrhagia, petechiae, nausea, hematemesis, melena and tremors; clinical laboratory examinations showed thrombocytopenia (<130,000 cells/mm^3), leukopenia (<3,900 cells/mm^3), and hematuria. Because physicians suspected BHF, patients received supportive therapy, including intravenous hydration, corticoids, antipyretic drugs, antimicrobial drugs, and blood transfusions from donors who had survived Machupo virus infection. Nonetheless, 2 of the patients died 3 and 4 days after admission.

In February 2008, at least 200 suspected new BHF cases (12 fatal) of BHF were reported to SEDES. A febrile hemorrhagic illness developed in a 19-year-old man from Huacaraje, Itenez Province (13°33′S, 63°45′W). On first examination at the Hospital Santa Maria Magdalena, the patient had fever, tremor, gingivorrhagia, petechiae, bruises, asthenia, and anorexia and was admitted with a presumptive diagnosis of BHF. Despite supportive treatment (including administration of plasma from a BHF survivor), his condition worsened; hematemesis, melena, hematochezia, hematuria, anuria, respiratory alkalosis, and metabolic acidosis developed in the patient, eventually resulting in death. A fifth case was detected in a 46-year-old man from San Ramon, Mamore Province (13°17′0″S, 64°43′0″W). A febrile hemorrhagic illness developed in the patient and he was admitted to the Hospital German Busch in Trinidad. The patient recently had been hired as a farm worker. When first seen by the attending physicians, he had fever, thrombocytopenia, leukopenia, petechias, tremors, gingivorrhagia, and dehydration, consistent with symptoms of BHF. The patient received hydration, corticoids, antipyretic therapy, and a plasma transfusion from a BHF survivor. The patient's condition improved and he was subsequently discharged from the hospital ≈10 days after admission.

Nineteen serum samples collected from suspected BHF patients, including the cases described above, were sent to Centro Nacional de Enfermedades Tropicales (Santa Cruz, Bolivia) and the US Naval Medical Research Center Detachment (Lima, Peru) for testing. Serum was injected into Vero and C6/36 cells; 10 days later, the cells were tested for flaviviruses, alphaviruses, and arenaviruses by indirect immunofluorescent assay and PCR. Five arenavirus isolates were obtained from the patients described in this report.

Viral RNA was extracted from the cell culture supernatant and the small (S) segment (≈3,200 bp) was amplified and sequenced. Phylogenetic analyses were conducted using the neighbor-joining and maximum likelihood program implemented in PAUP 4.0 software (Sinauer Associates, Inc., Sunderland, MA, USA). Sequence analyses confirmed the isolates as Machupo virus (Figure). Eight major Machupo phylogenetic lineages were described based on partial sequence of the nucleocapsid protein gene (*2*). We observed a similar tree topology based on the glycoprotein gene sequences (Figure). Two distinct lineages were distinguished among the isolates from the Itenez and Mamore provinces: V and VII and I and II, respectively. The recent isolates (2007–2008) from Magdalena and Huacaraje (Itenez Province) grouped within lineage V whereas the 2008 isolate from San

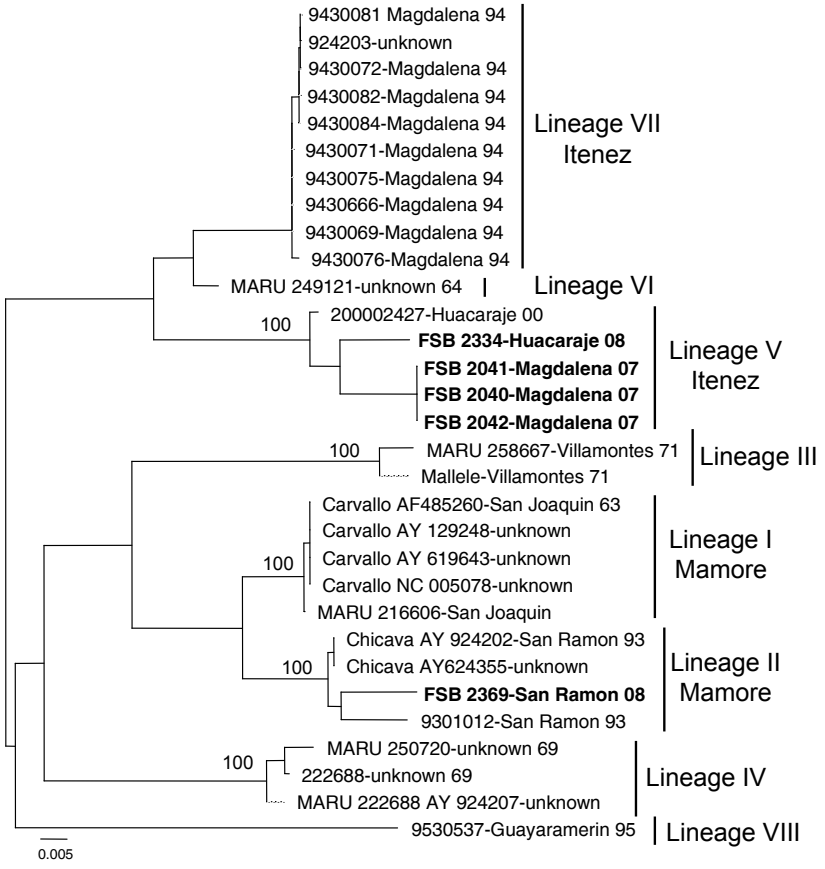

Figure. Neighbor-joining phylogenetic tree of Machupo virus derived from the glycoprotein precursor gene sequence. The neighbor-joining and maximum likelihood analyses yielded similar phylogenetic trees. **Boldface** indicates 2007–2008 isolates. Numbers indicate bootstrap values for 1,000 replicates. Scale bar indicates nucleotide substitutions per site.

Ramon (Mamore Province) belonged to lineage II. These isolates showed 10% nucleotide difference within the S segment and a 6% amino acid difference within the glycoprotein precursor gene. Similar genetic diversity has been described with Machupo virus and other arenaviruses (*2–4*). Sequences generated were deposited in GenBank (accession nos. FJ696411, FJ696412, FJ696413, FJ696414, and FJ696415).

It is not known whether lineage VII and I viruses continue to circulate or have been replaced by lineage V and II viruses, respectively. This study confirms the long-term maintenance of distinct phylogenetically forms of Machupo virus in a small area within Beni. Although the distribution of the Machupo virus rodent reservoir (*Calomys callosus*) extends beyond the geographic area of the Machupo cases described, factors that limit the endemic distribution of the virus remain unknown. However, population differences among *C. callosus* may account for the natural nidality of BHF (*5*). Studies are needed to fully identify and understand the ecology of the rodent reservoir and Machupo virus transmission.

Machupo virus continues to cause sporadic cases and focal outbreaks of BHF in Bolivia. We describe 5 confirmed human cases (3 fatal) of Machupo virus infection in Beni Department, Bolivia, an area in which BHF is endemic. That all 5 patients were farmers suggests their infections were probably acquired through occupational exposure. Although all the patients received plasma transfusion from patients who had survived BHF infection, 3 patients still died. An early diagnosis and the rapid administration of Machupo immune plasma before the hemorrhagic phase may increase the chance of survival, as has been observed with other arenavirus infections (*6–8*).

Acknowledgments

We thank Roxana Caceda and Juan Sulca for excellent technical assistance and the personnel of the Bolivian Ministry of Health for supporting our febrile illness surveillance study. Local activities were approved by the Ministry of Health of Bolivia and were developed by CENETROP personnel through local coordinators.

This study was funded by the United States Department of Defense Global Emerging Infections Systems Research Program, Work Unit No. 800000.82000.25GB.B0016.

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DOI: 10.3201/eid1509.090017

References

1. Johnson KM, Wiebenga NH, Mackenzie RB, Kuns ML, Tauraso NM, Shelokov A, et al. Virus isolations from human cases of hemorrhagic fever in Bolivia. Proc Soc Exp Biol Med. 1965;118:113–8.
2. Cajimat MN, Milazzo ML, Rollin PE, Nichol ST, Bowen MD, Ksiazek TG, et al. Genetic diversity among Bolivian arenaviruses. Virus Res. 2009;140:24–31. DOI: 10.1016/j.virusres.2008.10.016
3. Fulhorst CF, Charrel RN, Weaver SC, Ksiazek TG, Bradley RD, Milazzo ML, et al. Geographic distribution and genetic diversity of Whitewater Arroyo virus in the southwestern United States. Emerg Infect Dis. 2001;7:403–7.
4. Weaver SC, Salas RA, de Manzione N, Fulhorst CF, Travasos da Rosa AP, Duno G, et al. Extreme genetic diversity among Pirital virus (*Arenaviridae*) isolates from western Venezuela. Virology. 2001;285:110–8. DOI: 10.1006/viro.2001.0954
5. Salazar-Bravo J, Dragoo JW, Bowen MD, Peters CJ, Ksiazek TG, Yates TL. Natural nidality in Bolivian hemorrhagic fever and the systematics of the reservoir species. Infect Genet Evol. 2002;1:191–9. DOI: 10.1016/S1567-1348(02)00026-6
6. Fisher-Hoch SP, Tomori O, Nasidi A, Perez-Oronoz GI, Fakile Y, Hutwagner L, et al. Review of cases of nosocomial Lassa fever in Nigeria: the high price of poor medical practice. BMJ. 1995;311:857–9.
7. Maiztegui JI, Fernandez NJ, de Damilano AJ. Efficacy of immune plasma in treatment of Argentine haemorrhagic fever and association between treatment and a late neurological syndrome. Lancet. 1979;2:1216–7. DOI: 10.1016/S0140-6736(79)92335-3
8. Enria DA, Briggiler AM, Sanchez Z. Treatment of Argentine hemorrhagic fever. Antiviral Res. 2008;78:132–9. DOI: 10.1016/j.antiviral.2007.10.010

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Relapsing Fever Spirochete in Seabird Tick, Japan

To the Editor: Tick-borne relapsing fever (TBRF) is caused by infection with spirochetes belonging to the genus *Borrelia*. We previously reported a human case of febrile illness suspected to be TBRF on the basis of serologic examination results; the vector most likely was a genus *Carios* tick that had fed on a seabird colony (*1*). However, surveillance of ticks in the area did not identify *Borrelia* spp. in any of the *Carios* ticks sampled (2). In 2007 and 2008, a borreliosis investigation was conducted on Kutsujima Island (35.71′N, 135.44′E) because

a bird-associated tick, genus *Carios*, inhabits this island. During the investigation, 77 *Carios* ticks (55 nymphs, 11 adult males, and 11 adult females) were collected from colonies of seabirds: Swinhoe's storm petrel (*Oceanodroma monorhis*) and streaked shearwater (*Calonectris leucomelas*). Identification of tick species as *C. sawaii* was based on tick morphology and *rrs* gene sequence analysis of the tick mitochondrion DNA (*2*). Total DNA was extracted from the ticks by using a DNeasy Tissue Kit (QIAGEN, Germantown, MD, USA). For the detection of *Borrelia* DNA, PCR designed was based on the flagellin gene (*flaB*) according to Sato et al. (*3*). To check for contamination and amplicon carryover, we used blank tubes as a negative control for each experiment. Of 77 *C. sawaii* ticks that were positive by PCR of tick genes (*2*), 25 (14 nymphs, 6 adult males, 5 adult females) were positive for *Borrelia* DNA by PCR of *flaB*.

To characterize the *Borrelia* spp., we sequenced amplified fragments of the *flaB* gene and the 16S ribosomal RNA (*16SrRNA*) gene of *Borrelia* spp. in a tick and compared the results with those of representative *Borrelia* spp. The primers BflaPBU and BflaPCR (*3*) for *flaB* and the 4 PCR primers (online Technical Appendix, available from www.cdc.gov/EID/content/15/9/1528-Techapp.pdf) for *16SrRNA* were used for direct sequencing and/or amplification. DNA sequence (GenBank accession no. AB491928) of a 294-bp amplified fragment of *flaB* showed the following nucleotide similarities with those of *Borrelia* spp.: *B. turicatae* (98.98%), *B. parkeri* (98.30%), *Borrelia* sp. Carios spiro-1 (98.64%), and *Borrelia* sp. Carios spiro-2 (98.30%). DNA sequence (GenBank accession no. AB491930) of a 1,490-bp amplified fragment of *16SrRNA* showed the following nucleotide similarities with those of *Borrelia* spp.: *B. turicatae* (99.60%), *B. parkeri* (99.53%), and *Borrelia* sp. Carios spiro-2 (99.45%). *Borrelia* sp. Carios spiro-1 and Carios spiro-2, which were recently identified in *C. kelleyi* in the United States, have been classified into TBRF *Borrelia* (*4,5*). The *Borrelia* sp. found in this study, designated as *Borrelia* sp. K64, was closely related to *B. turicatae* but was distinct from other TBRF *Borrelia* spp. (online Technical Appendix).

To observe *Borrelia* spp. in tick tissues, we performed an indirect fluorescence assay (IFA) according to methods described by Fisher et al. (6), with minor modifications. A tick that was negative by PCRs of *flab* and *16SrRNA* was used as a negative control. The IFA of the tick salivary gland and midgut was conducted by using acetone for fixation, goat anti-*Borrelia* sp. polyclonal immunoglobulin (Ig) G (1:100; KPL, Inc., Gaithersburg, MD, USA) as the primary antibody, and Alexa fluor 488-labeled rabbit antigoat IgG (1:200, Invitrogen, Carlsbad, CA, USA) as the secondary antibody. Our analysis showed a spirochete, which was stained by anti-*Borrelia* spp. antibody, in salivary gland and midgut tissue (online Technical Appendix). However, no spirochetes were detected by IFA in the negative control (data not shown).

We also attempted to isolate *Borrelia* spp. from tick specimens by using Barbour-Stoenner-Kelly medium (*7*). The motility of *Borrelia*-like organisms in the medium was initially observed by using dark-field microscopy. The *Borrelia*-like organisms were identified as *Borrelia* sp. K64 by sequencing of PCR-amplified fragments of *flaB* and *16SrRNA* genes from the cultured medium. However, these *Borrelia* organisms were found for only 2 weeks after inoculation (data not shown).

The vertebrate reservoir hosts of TBRF *Borrelia* are usually rodents but can be a variety of other animals (*8*). Although competence as a reservoir has not been determined for birds, infection of an owl with a TBRF *Borrelia* sp. has been reported (*9*). The vertebrate host of the spirochete has not yet been determined. Given our results, it is possible that seabirds are potential vertebrate hosts for *Borrelia* spp.

In Japan, relapsing fever is a neglected infectious disease because it was not reported during 1956–1998 (*10*). In this study, we detected a *Borrelia* sp. in *C. sawaii*, and the spirochete we characterized is closely related to *B. turicatae*. Although the human health implications of infections caused by *Borrelia* spp. are not yet known, the findings from this study should contribute to the epidemiologic investigation of TBRF in Japan.

Acknowledgements

We thank Kiyotaka Karino for the tick collection and Eri Watanabe, Manabu Ato, and Norio Ohashi for the imaging analysis. We are also grateful to Jun Ohnishi for technical information regarding tick dissection.

This study was supported by the Global Environment Research Fund (F-3 and F-081, leader: K. Goka) 1 of the Ministry of the Environment, Japan 2008, and by a grant for Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labor, and Welfare of Japan.

Ai Takano, Maki Muto, Akiko Sakata, Yumiko Ogasawara, Shuji Ando, Nozomu Hanaoka, Miyako Tsurumi, Fumio Sato, Noboru Nakamura, Hiromi Fujita, Haruo Watanabe, and Hiroki Kawabata

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DOI: 10.3201/eid1509.090459

References

1. Tsurumi M, Kawabata H, Sato F. Present status and epidemiological investigation of *Carios* (*Ornithodoros*) *capensis* in the colony of the black-footed albatross Diomedea nigripes on Tori-shima, Izu Islands, Japan [in Japanese]. Journal of the Yamashina Institute for Ornithology. 2002;10:250–6.
2. Kawabata H, Ando S, Kishimoto T, Kurane I, Takano A, Nogami S, et al. First detection of Rickettsia in soft-bodied ticks associated with seabirds, Japan. Microbiol Immunol. 2006;50:403–6.
3. Sato Y, Konishi T, Hashimoto Y, Takahashi H, Nakaya K, Fukunaga M, et al. Rapid diagnosis of Lyme disease: flagellin gene–based nested polymerase chain reaction for identification of causative *Borrelia* species. Int J Infect Dis. 1997;2:64–73. DOI: 10.1016/S1201-9712(97)90084-9
4. Loftis AD, Gill JS, Schriefer ME, Levin ML, Eremeeva ME, Gilchrist MJ, et al. Detection of *Rickettsia*, *Borrelia*, and *Bartonella* in *Carios kelleyi* (Acari: Argasidae). J Med Entomol. 2005;42:473–80. DOI: 10.1603/0022-2585(2005)042[0473:DORBAB]2.0.CO;2
5. Gill JS, Ullmann AJ, Loftis AD, Schwan TG, Raffel SJ, Schrumpf ME, et al. Novel relapsing fever spirochete in bat tick. Emerg Infect Dis. 2008;14:522–3. DOI: 10.3201/eid1403.070766
6. Fisher MA, Grimm D, Henion AK, Elias AF, Stewart PE, Rosa PA, et al. *Borrelia burgdorferi* sigma54 is required for mammalian infection and vector transmission but not for tick colonization. Proc Natl Acad Sci U S A. 2005;102:5162–7. DOI: 10.1073/pnas.0408536102
7. Barbour AG. Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med. 1984;57:521–5.
8. Barbour AG. Relapsing fever. In: Goodman JL, Dennis DT, Sonenshine DE, editors. Tick-borne diseases of humans. Washington: ASM Press; 2005. p. 268–91.
9. Thomas NJ, Bunikis J, Barbour AG, Wolcott MJ. Fatal spirochetosis due to a relapsing fever-like *Borrelia* sp. in a northern spotted owl. J Wildl Dis. 2002;38:187–93.
10. Ministry of Health, Labour and Welfare, Japan. MHLW statistical database; 1998 [in Japanese] [cited 2009 Jun 23]. Available from http://wwwdbtk.mhlw.go.jp/toukei/data/210/1998/toukeihyou/0002222/t0033267/hyo02_001.html

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Backyard Raccoon Latrines and Risk for *Baylisascaris procyonis* Transmission to Humans

To the Editor: Raccoons (*Procyon lotor*) are abundant in urban environments and carry a variety of diseases that threaten domestic animals (*1*) and humans (2,*3*). A ubiquitous parasite of raccoons, *Baylisascaris procyonis* causes a widely recognized emerging zoonosis, baylisascariasis (*3*). Although only 14 human cases of severe *B. procyonis* encephalitis have been reported over 30 years (*4*), prevention is still a priority for public health and wildlife officials because of the seriousness of the resulting neurologic disease (*5*).

Raccoons prefer to defecate at latrines they create. Infected animals shed ≈20,000 eggs/g of feces (*3*), so latrines serve as the foci of parasite transmission (*6*). When latrines occur in close proximity to humans, the risk for zoonotic transmission increases (*2*). Because *B. procyonis* are transmitted by the fecal–oral route, young children have the greatest risk for zoonotic infection because of their tendency to put objects into their mouths (*1*,*2*). Many human cases have occurred in environments where latrines were near children's play areas. Our objective was to determine which factors encourage raccoons to create latrines in human habitats. This information will allow public health officials and wildlife managers to develop strategies to educate the public and to ultimately prevent zoonotic transmission.

We surveyed 119 backyards for raccoon latrines in the suburbs of Chicago, Illinois, USA, near the Ned Brown Forest Preserve (n = 38; 42°01′55.05″N, 88°00′00.62″W, Cook County) and Lincoln Marsh (n = 81; 41°51′4.54″N, 88°5′39.019″W, DuPage County). Yards were selected on the basis of proximity to forest preserves and willingness of homeowners to participate in the study. We located latrines by systematically searching yards, giving special attention to horizontal substrates, such as piles of wood and the bases of large trees (*6*). We removed all fecal material to test for *B. procyonis* and stored it in plastic bags at –20°C until analysis. Composite samples that were at least 2 g underwent fecal flotation in Sheather solution (*7*) (at least 1 g of every fecal deposit at a latrine) (n =131). We identified *B. procyonis* eggs by microscopic examination on the basis of their size and morphologic appearance (*2*). Multiple slides were examined for ≈10% of the samples (randomly selected) to validate our results. Prevalence was considered the proportion of positive samples from all sampled yards.

Each yard was additionally surveyed for potential latrine substrates (*8*) and factors believed to attract or deter raccoons. The distance of each yard from the nearest forested habitat was calculated by using ArcGIS 9.0 (Geographic Information Systems, Redlands, CA, USA). We used homogeneity tests to identify differences in the proportion of yards with latrines present and to compare the prevalence of *B. procyonis* between study areas. Logistic regression and odds ratios were used to evaluate a main effect model composed of 10 yard attributes, including the presence of a pet, birdfeeders, garbage cans, and sandboxes, and to evaluate a simplified model in which attributes were combined to reflect the presence of food and latrine substrates, such as pet food, birdfeed, garbage and piles of wood or logs, respectively.

Latrines occurred in 61/119 yards (51%; 95% confidence interval [CI] 0.42%–0.60%). There was no significant difference in the proportion of backyards with latrines in proximity to Ned Brown (23/38, 82%) and Lin-

coln Marsh (38/81, 46%). The number of latrines per backyard ranged from 1 to 6 ($\chi = 2.15$). *B. procyonis* eggs were found at 14/61 latrines sampled (23%; 95% CI 12%–34%), and no significant difference in prevalence was found between the Ned Brown (6/23, 26%; 95% CI 8%–44%) and Lincoln Marsh areas (8/38, 21%; 95% CI 8%–34%).

Evaluation of the main effect model identified a decreasing probability of latrine occurrence with increasing distance from the nearest forested area and the presence of an outdoor pet, although these relationships were only marginally significant ($p = 0.07$ and 0.08, respectively). No other variables were closely associated with the presence of raccoon latrines ($p>0.20$). When evaluated alone, distance from the forest preserve was significantly related to latrine occurrence ($p = 0.03$); probability decreased with increasing distance. Evaluation of the simplified model identified a weakly positive association with the presence of a food source ($p = 0.09$) and no association with the presence of latrine substrate ($p = 0.35$). Although the findings were not statistically significant, raccoon latrines did appear to be associated with the availability of a food source such as bird feed (odds ratio [OR] 1.9, 95% CI 0.9–4.1); the presence of an outdoor pet (OR 0.27, 95% CI 0.06–1.2) and increasing distance from the nearest forested area reduced the likelihood of latrines. No other variables were associated with the presence of raccoon latrines; however, low statistical power may have precluded adequate assessment.

Our results suggest that when humans live close to protected forests or natural areas, they are more likely to attract raccoons into their yards. In addition, anthropogenic food sources such as pet food, garbage, and bird feed may increase the likelihood that a raccoon will create a latrine, and the presence of outdoor pets appears to be a deterrent. In areas of high raccoon density, these attractants should be removed. Homeowners with small children should remove latrines as quickly as they are discovered (*2*). The risk of children acquiring potentially fatal baylisascariasis can be reduced if parents understand how to reduce the likelihood that children will come into contact with raccoon latrines.

Acknowledgments

The authors thank the Cook County Forest Preserve District, the Elk Grove Village, the Illinois Health Department, and the numerous cooperating homeowners in both Elk Grove Village and Wheaton, Illinois.

This study was supported by the Wheaton College Alumni Association, the Aldeen Fund, and the Science Division of Wheaton College.

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DOI: 10.3201/eid1509.090128

References

1. Kazacos KR. Protecting children from helminthic zoonoses. Contemp Pediatr. 2000;17:1–24.
2. Kazacos KR. *Baylisascaris procyonis* and related species. In: Samuel WM, Pybus MJ, Kocan AA, editors. Parasitic diseases of wild mammals. Ames (IA): Iowa State University Press; 2001.
3. Sorvillo F, Ash LR, Berlin OGW, Yatabe J, Degiorgio C, Morse SA. *Baylisascaris procyonis:* an emerging helminthic zoonosis. Emerg Infect Dis. 2002;8:355–9.
4. Pai PJ, Blackburn BG, Kazacos KR, Warrier RP, Begue RE. Full recovery from *Baylisascaris procyonis* eosinophilic meningitis. Emerg Infect Dis. 2007;13: 928–30.
5. Gavin PJ, Kazacos KR, Shulman ST. Baylisascariasis. Clin Microbiol Rev. 2005;18:703–18. DOI: 10.1128/CMR.18.4.703-718.2005
6. Page LK, Swihart RK, Kazacos KR. Raccoon latrine structure and its potential role in transmission of *Baylisascaris procyonis* to vertebrates. Am Midl Nat. 1998;140:180–5. DOI: 10.1674/0003-0031(1998)140[0180:RLSAIP]2.0.CO;2
7. Sloss MW, Kemp RL, Zajac AM. Veterinary clinical parasitology. Ames (IA): Iowa State University Press; 1994.
8. Page LK, Swihart RK, Kazacos KR. Implications of raccoon latrines in the epizootiology of baylisascariasis. J Wildl Dis. 1999;35:474–80.

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Reemergence of Strongyloidiasis, Northern Italy

To the Editor: Strongyloidiasis is a helminth infection caused by *Strongyloides stercoralis,* a nematode ubiquitous in tropical and subtropical countries and occasionally reported in temperate countries, including Italy (*1*). Sources of infection are filariform strongyloid larvae present in soil contaminated by infected feces; the larvae penetrate through the skin of a human host. After the first life cycle, a process of autoinfection begins, which persists indefinitely in the host if the infection is not effectively treated. The infection can remain totally asymptomatic for many years or forever or cause cutaneous (itching and rash), abdominal (epigastric pain, pseudoappendicitis, diarrhea), respiratory (cough, recurrent asthma), and systemic (weight loss, cachexia) symptoms that can be enervating. More importantly, when host immunity is impaired because of

a concurrent disease or immunosuppressive therapy (including corticosteroids, sometimes used to treat symptoms of the unrecognized infection or the concurrent eosinophilia), disseminated strongyloidiasis may occur (*2*–*4*), causing a massive and almost invariably fatal invasion of virtually all organs and tissues by filariform larvae and even adult worms (Figure), often combined with bacterial superinfection. This complication is believed to be rare but is probably underestimated because of the extreme variability of the clinical presentation.

Although strongyloidiasis can be suspected in the presence of symptoms or eosinophilia (which is frequent but not mandatory), the low sensitivity of direct diagnostic methods often lets the disease go unrecognized (*5*–*7*). By far the most sensitive diagnostic tools are serologic tests: sensitivity and specificity of indirect fluorescent antibody test (IFAT) (in-house produced IFAT) are 97.4% and 97.9%, respectively, at a dilution ≥1/20, and 70.5% and 99.8% at a dilution ≥1/80 (*6*). A suspected case is defined by a positive antibody titer ≥20 (IFAT); a case is confirmed by a positive direct test result (culture in agar being the most sensitive direct technique) or by a positive antibody titer ≥80 (*6*). Despite some anecdotal reports on the presence of strongyloidiasis in Italy (*1*,*6*), reliable information about the real prevalence of the infection is lacking. After seeing several patients affected by the disease, 1 of whom died because of dissemination (Z. Bisoffi, unpub. data), we decided to carry out a preliminary rapid assessment of the extent of the problem in elderly patients with eosinophilia.

During a 4-month period, from February through May 2008, every patient born in 1940 or earlier who came to the clinical laboratories of 2 contiguous health districts in northern Italy (Mantova, Lombardy Region, and Legnago, Veneto Region) for a diagnostic blood test (hematocrit and leukocyte count/formula) for whatever reason and having a eosinophil count >500 cells/μL was asked to join the study. This study was the pilot phase of a larger, multicentered study, which obtained formal approval from the Ethical Committee of Sacro Cuore Hospital of Negrar, Verona. Informed consent was required of each patient. Of the 132 patients eligible for inclusion (mean age 76.4 years, range 68–90 years, male:female ratio 1.6), none refused to give informed consent. Serum specimens were subjected to the IFAT for *S. stercoralis* at the Sacro Cuore Hospital Centre for Tropical Diseases.

Unexpectedly, we found that 37 (28%) of 132 patients were positive, with titers ranging between 20 and ≥320 (and ≥80 in most cases). However, caution should be exercised in interpreting the results because the patients may not be representative of the general population. Moreover, our results are based on an indirect (although highly sensitive and specific) test. Because the reported cases involve only a few patients every year (of whom some are anecdotally reported as dying from the infection, usually unpublished), we suspect that most strongyloidiasis cases remain undetected.

If relevant transmission still exists in the area, it is unknown but is unlikely because of the improvement of hygienic conditions in the past 5 decades. Reports of the infection in children or young adults with no travel history outside Italy are lacking. Strongyloidiasis in the elderly is therefore most likely to result from an infection that occurred much earlier in life, either in infancy or at a young age, while walking or working barefoot in agricultural fields. The long persistence is the consequence of the autoinfection cycle typical of this parasite as described above. The result is an important and unrecognized public health problem affecting the geriatric population of northern Italy. These preliminary results confirm the need for the already planned, multicentered study involving a larger sample and a wider geographic area.

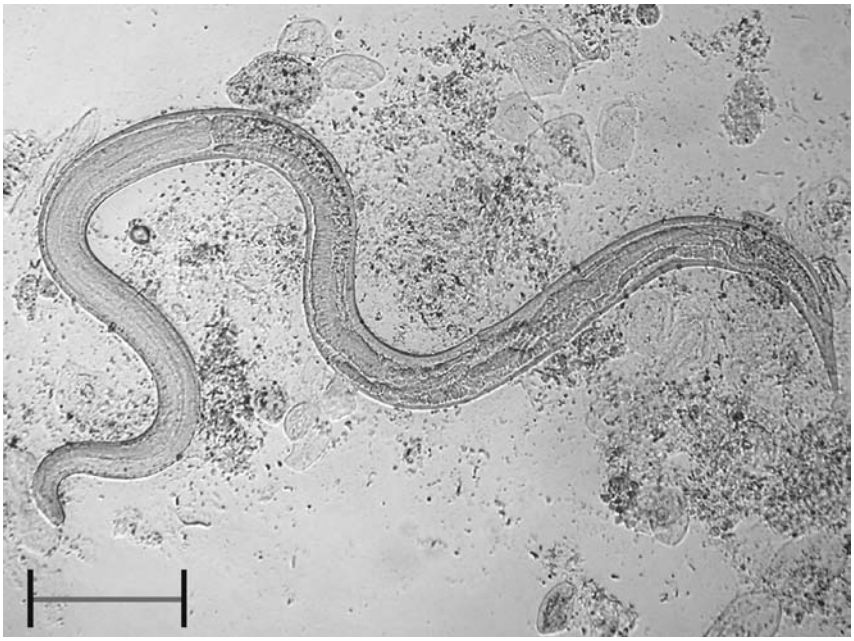

Figure. Adult female of *Strongyloides stercoralis* collected in bronchial fluid of a patient with disseminated disease. Scale bar = 400 μm. A color version of this figure is available online (www.cdc.gov/EID/content/15/9/1531-F.htm).

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DOI: 10.3201/eid1509.090191

References

1. Pirisi M, Salvador E, Bisoffi Z, Gobbo M, Smirne C, Gigli C, et al. Unsuspected strongyloidiasis in hospitalised elderly patients with and without eosinophilia. Clin Microbiol Infect. 2006;12:787–92.
2. Scowden EB, Schaffner W, Stone WJ. Overwhelming strongyloidiasis: an unappreciated opportunistic infection. Medicine. 1978;57:527–44. DOI: 10.1097/00005792-197811000-00004
3. Fardet L, Genereau T, Poirot JL, Guidet B, Kettaneh A, Cabane J. Severe strongyloidiasis in corticosteroid-treated patients: case series and literature review. J Infect. 2007;54:18–27. DOI: 10.1016/j.jinf.2006.01.016
4. Boscolo M, Bisoffi Z. Dissemination: the fatal risk for a missed diagnosis of *Strongyloides stercoralis* infection. J Infect. 2007;55:284–5. DOI: 10.1016/j.jinf.2007.01.009
5. Siddiqui AA, Berk SL. Diagnosis of *Strongyloides stercoralis* infection. Clin Infect Dis. 2001;33:1040–7. DOI: 10.1086/322707
6. Boscolo M, Gobbo M, Mantovani W, Degani M, Anselmi M, Badona Monteiro G, et al. Evaluation of an indirect immunofluorescence assay for strongyloidiasis as a tool for diagnosis and follow-up. Clin Vaccine Immunol. 2007;14:129–33. DOI: 10.1128/CVI.00278-06
7. Loutfy MR, Wilson M, Keystone JS, Kain KC. Serology and eosinophil count in the diagnosis and management of strongyloidiasis in a non-endemic area. Am J Trop Med Hyg. 2002;66:749–52.

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Salmonella enterica Serovar Typhi with CTX-M β-Lactamase, Germany

To the Editor: Infection with *Salmonella enterica* serovar Typhi, the causative agent of typhoid fever, is an acute systemic illness with a high proportion of illness and deaths, especially in developing countries. In Europe, *S. enterica* ser. Typhi infections occur among travelers returning from disease-endemic areas. After emergence of multidrug-resistant *S. enterica* ser. Typhi strains that confer resistance to chloramphenicol, trimethoprim, and ampicillin, quinolones have become the primary drugs for treatment (*1*). Here we report the isolation of CTX-M–producing *S. enterica* ser. Typhi in Germany.

We isolated *S. enterica* ser. Typhi from blood and feces specimens from a 30-year-old Iraqi woman who was admitted to the hospital in Cologne in August 2008. The patient was febrile, dizzy, and had epigastric pain and headache. The symptoms began 2 weeks earlier, after she had returned from a month-long visit to her relatives in Sulaymaniya, the capital of As Sulaymaniyah Governorate in the northeastern Iraqi Kurdistan region. The interview indicated that the same symptoms had developed in other family members in Iraq. The patient was treated successfully with meropenem (1 g 3×/day) for 2 weeks, and no relapse was observed in a follow-up period of 6 months.

The isolated strain was identified as *S. enterica* ser. Typhi with the VITEK2 system (VITEK2 GN-card; bioMérieux, Brussels, Belgium) and by slide agglutination with *Salmonella* antisera (SIFIN, Berlin, Germany) in accordance with the Kauffmann-White scheme. By using Vi-phage typing according to the International Federation for Enteric Phage Typing (L.R. Ward, pers. comm.), the strain was classified as *S. enterica* ser. Typhi Vi-phage type E9. Antimicrobial drug susceptibilities were determined according to the guidelines of the Clinical Laboratory Standards Institute with the VITEK2 AST-N021 card and Etest (bioMérieux). The extended-spectrum β-lactamase (ESBL) phenotype was confirmed with a combined disk diffusion test (MASTDISCS ID, Mast Diagnostica GmbH, Germany). PCR and sequence analyses were performed with universal primers for the ESBL genes $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} as described previously (*2*). Primer CTX-M-F 5′-GTTCGTCTCTTCCAGAATAAGG-3′ and primer CTX-M-R 5’-CAGCACTTTTGCCGTCTAAG-3′ were used for sequencing the entire $bla_{\text{CTX-M}}$ gene. Investigation of the CTX-M environment was performed with primers IS*26*-F (5′-GCCCTGGTAAGCAGAGTTTTTG-3′) and IS26-CTX-R (5′-ACAGCGGCACACTTCCTAAC-3′). The presence of plasmid-mediated quinolone resistance genes (*qnr*) was determined by PCR and sequencing of *qnrB* (*3*), *qnrS* (primer F, 5′-CGGCACCACAACTTTTCAC-3′; primer R, 5′-CAACAATACCCAGTGCTTCG-3′), and *qnrA* (primer F, 5′-ATTTCTCACGCCAGGATTTG-3′; primer R, 5′-CGGCAAAGGTTAGGTCACAG-3′). In addition, the nucleotide sequences of the quinolone resistance-determining regions of the *gyrA*, *gyrB*, *parC,* and *parE* genes were determined as previously described (*4*). Transfer of β-lactam resistance was tested by broth mating assays with a sodium azide–

resistant *Escherichia coli* J53 recipient. Selection of transconjugants was performed on Mueller-Hinton agar plates that contained sodium azide (200 μg/mL) and ampicillin (100 μg/mL). We isolated the plasmid DNA of donor and transconjugants using the QIAGEN Plasmid Mini Kit (QIAGEN, Hilden, Germany).

Phenotypically, the strain was resistant to ampicillin, ampicillin/sulbactam, piperacillin, cefotaxime, ceftazidime, cefepime, chloramphenicol, streptomycin, trimethoprim/sulfamethoxazole, azithromycin, and nalidixic acid. A reduced susceptibility to ciprofloxacin was detected (MIC_{CIP} = 1 μg/mL). The isolate was susceptible to imipenem, meropenem, gentamicin, tobramycin, and amikacin. PCR and sequence analyses displayed the presence of $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$ and the *qnrB2* gene. We found an amino acid substitution in *gyrA* gene (83-Ser→Phe). No mutations were identified in the *gyrB*, *parC*, and *parE* genes. Sequencing of the insertion element (IS)*26*-F/R amplification product showed the location of IS*26 transposase A* gene *(tnpA),* followed by a truncated IS*Ecp1* mobile element upstream of the $bla_{CTX\text{-}M\text{-}15}$ gene. By conjugation, 1 plasmid of ≈50 kbp was successfully transferred into an *E. coli* J53 recipient (Figure). PCR-based replicon typing (*5*) showed an IncN–related plasmid. The *E. coli* J53 transconjugant mediated resistance to ampicillin, cefotaxime, ceftazidime, cefepime, trimethoprim/sulfamethazole, nalidixic acid and showed reduced susceptibility to ciprofloxacin (MIC = 0.5 μg/mL). Also, in the transconjugant, the $bla_{CTX\text{-}M\text{-}15}$ and *qnrB2* genes were identified by PCR.

ESBL-producing non-Typhi serotypes of *S. enterica* are an increasing problem worldwide. In Europe and Asia, CTX-M-group ESBLs are prevalent in *S. enterica,* and in North America, domestically acquired CTX-M ESBLs were recently identified in *S. enterica* ser. Typhimurium (*6*). In *S. enterica* ser. Typhi, reports of ESBLs have been rare. The CTX-M-15 type that we found has been reported only once previously in *S. enterica* ser. Typhi from Indian patients hospitalized in Kuwait (*7*). In addition to cephalosporin resistance mediated by ESBLs, the reduced susceptibility to quinolones in *S. enterica* is of concern. In the study isolate, this reduced susceptibility was due to a known mutation 83-Ser→Phe in *gyrA* (*8*) and the acquisition of a *qnB2* gene. Plasmid-mediated Qnr determinants have been identified in *S. enterica* of different non-Typhi serovars (*9*), whereas in *S. enterica* ser. Typhi, only mutations in gyrase and topoisomerase genes leading to quinolone resistance had been observed previously (*8*).

In our isolate of *S. enterica* ser. Typhi that contained $bla_{CTX\text{-}15}$ and *qnrB2,* resistance to cephalosporins as well as the reduced quinolone susceptibility was easily transferable by conjugation into *E. coli.* This occurrence is alarming because the dissemination of such strains with acquired resistances will further limit the therapeutic options for treatment of typhoid fever.

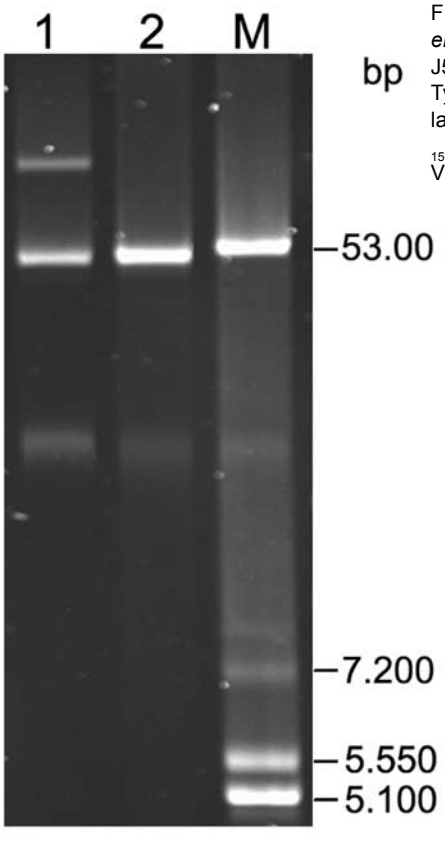

Figure. Plasmids isolated from *Salmonella enterica* serovar Typhi and *Escherichia coli* J53 transconjugant. Lane 1, *S. enterica* ser. Typhi 218/08 ($bla_{CTX\text{-}M\text{-}15}$ + $bla_{TEM\text{-}1}$ + *qnrB2*); lane 2, *E. coli* J53 transconjugant ($bla_{CTX\text{-}M\text{-}15}$ + *qnrB2*); lane M, plasmid marker *E. coli* V517.

Acknowledgments

We thank George A. Jacoby for providing the *E. coli* J53 Azi^r strain, Alessandra Carattoli for supplying Inc group reference strains, and Jean M. Whichard for critical reading. We extend special thanks to Dagmar Busse and Bettina Leiste for phage typing and phenotypical analysis.

This work was funded by the Ministry of Health, Germany.

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References

1. Pokharel BM, Koirala J, Dahal RK, Mishra SK, Khadga PK, Tuladhar NR. Multidrug-resistant and extended-spectrum β-lactamase (ESBL)–producing *Salmonella enterica* (serotypes Typhi and Paratyphi A) from blood isolates in Nepal: surveillance of resistance and a search for newer alternatives. Int J Infect Dis. 2006;10:434–8. DOI: 10.1016/j.ijid.2006.07.001
2. Gröbner S, Linke D, Schütz W, Fladerer C, Madlung J, Autenrieth IB, et al. Emergence of carbapenem–non-susceptible extended-spectrum β-lactamase (ESBL)–producing *Klebsiella pneumoniae* isolates at the university hospital of Tübingen, Germany. J Med Microbiol. 2009; 58:912–22.
3. Jacoby GA, Walsh KE, Mills DM, Walker VJ, Oh H, Robicsek A, et al. qnrB, another plasmid-mediated gene for quinolone resistance. Antimicrob Agents Chemother. 2006;50:1178–82. DOI: 10.1128/AAC.50.4.1178-1182.2006
4. Giraud E, Brisabois A, Martel JL, Chaslus-Dancla E. Comparative studies of mutations in animal isolates and experimental in vitro- and in vivo-selected mutants of *Salmonella* spp. suggest a counterselection of highly fluoroquinolone-resistant strains in the field. Antimicrob Agents Chemother. 1999;43:2131–7.
5. Carattoli A, Miriagou V, Bertini A, Loli A, Colinon C, Villa L, et al. Replicon typing of plasmids encoding resistance to newer beta-lactams. Emerg Infect Dis. 2006;12:1145–8.
6. Sjölund M, Yam J, Schwenk J, Joyce K, Medalla F, Barzilay E, et al. Human *Salmonella* infection yielding CTX-M beta-lactamase, United States. Emerg Infect Dis. 2008;14:1957–9. DOI: 10.3201/eid1412.080494
7. Rotimi VO, Jamal W, Pal T, Sovenned A, Albert MJ. Emergence of CTX-M-15 type extended-spectrum beta-lactamase-producing *Salmonella* spp. in Kuwait and the United Arab Emirates. J Med Microbiol. 2008;57:881–6. DOI: 10.1099/jmm.0.47509-0
8. Capoor MR, Nair D, Walia NS, Routela RS, Grover SS, Deb M, et al. Molecular analysis of high-level ciprofloxacin resistance in *Salmonella enterica* serovar Typhi and *S.* Paratyphi A: need to expand the QRDR region? Epidemiol Infect. 2009;137:871–8.
9. Gay K, Robicsek A, Strahilevitz J, Park CH, Jacoby G, Barrett TJ, et al. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. Clin Infect Dis. 2006;43:297–304. DOI: 10.1086/505397

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Gordonia sputi Bacteremia

To the Editor: In November 2007, a 69-year-old man with fever was hospitalized at the Northern Hospital in Marseilles, France. He also had diabetes, high blood pressure, and alcohol and tobacco addictions. In September 2007, he had received a diagnosis of laryngeal cancer, which required 2 chemotherapy treatments through a central venous catheter (CVC), the second of which he had received 6 days before his November visit. Prostatic cancer, diagnosed 1.5 years earlier, had been treated by radiotherapy. At the time of the November admission, he had leukopenia (1.77×10^9 leukocytes/L with 0.49×10^9 polymorphonuclear cells/L) and an elevated C-reactive protein level (151 mg/L). The patient was admitted with a preliminary diagnosis of drug-induced febrile granulocytosis; the origin of his fever remained unclear. Blood for culture was first collected from a peripheral vein and on the next day was collected from a peripheral vein and from the CVC. Gram-positive rods grew in the aerobic bottle from the CVC sample. The microorganism was identified by biochemical tests using API Coryne strip (bioMérieux, Marcy-l'Etoile, France) as *Rhodococcus* spp. (94% similarity). The day after hospital admission, the patient was empirically treated with intravenous ticarcillin-clavulanate, 5 g 3×/day; ciprofloxacin, 200 mg 2×/day; and granulocyte colony–stimulating factor. One day later, fever resolved and the polymorphonuclear cell count was within normal limits. Oral antimicrobial drug therapy was continued for 1 week.

Bacterial identification of the strain was performed by 16S rRNA sequencing. We obtained a 1,464-bp sequence, which was found to differ at only 2 nt positions from that of *Gordonia sputi* (GenBank accession no. X80634). We concluded that our patient had catheter-related bacteremia caused by *G. sputi* because he was immunocompromised and had a CVC. We ruled out a contaminant because the organism did not belong to the normal flora of human skin and because fever resolved after treatment with antimicrobial drugs.

The genus *Gordona* was first described in 1971, for coryneform bacteria isolated from sputum of patients with pulmonary disease or from soil (*1*). It is a member of the mycolic acid–containing group consisting of genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella*. The genus has been revised several times by rearrangements with the genera *Rhodococcus* and *Nocardia*, and the name *Gordona w*as changed to *Gordonia* in 1997. The genus belongs to suborder *Corynebacterineae* within the order *Actinomycetales* and currently contains 27 recognized species; only 7 have been described in human disease. Species are identified by molecular analysis.

Gordonia spp. cause a wide spectrum of disease in humans (*2–10;* Table). Neurologic and vascular infections in immunocompromised and immunocompetent patients have been reported. Cutaneous and respiratory infections, otitis externa, osteitis, and arthritis have reportedly occurred only in immunocompetent patients. Bacteria have most often been isolated from blood samples. Bacteremia has started from underlying disease such as a sequestrated lung (*4*) or acute cholecystis (*5*) or has been related to coronary artery surgery (*2*) and frequently to CVCs (*2,3,6*). Catheter removal has been recommended for treatment of

Table. Summary of clinical reports involving *Gordonia* spp.*

Gordonia sp.	Clinical findings, by system					
	Vascular	Cutaneous	ENT	Nervous	Osteoarticular	Respiratory
G. rubripertincta	Bacteremia (CVC) (*2*)					Lung infection (*2*)
G. terrae	Bacteremia (cholecystitis) (*5*); bacteremia (CVC) (*2*); bacteremia (CVC) (*6*)†	Granulomatous skin lesion (*2*); palpebral abscess (*9*); granulomatous mastitis (*7*); mycetoma of the hand (*9*)		Meningitis, brain abscess (*2*); brain abscess (*2*)†		
G. bronchialis	Bacteremia (sequestrated lung) (*4*)	Recurrent breast abscess (*7*)		Ventriculitis (intraventricular shunt) (*6*)	Sternal wound infections (*2*)	
G. polyisoprenivorans	Endocarditis (CVC) (*3*);† bacteremia (CVC) (*3*)†					
G. otitidis	Bacteremia (CVC) (*6*)		Otitis externa (*6*)			Bronchitis (*8*)
G. sputi	Bacteremia (CVC) (*2*)†; endocarditis (CVC) (*2*); mediastinitis (surgery) (*2*)					
G. araii					Arthritis (bioabsorbable tapered screw) (*10*)	

*ENT, ear, nose, throat; CVC, central venous catheter. Patients were immunocompetent unless otherwise noted. When infection was associated with a medical device, the device is listed in parentheses.
†Immunocompromised patient.

Gordonia spp. infections in children (*6*), but the recommendation varied for adults. Six cases of infection in children have been described (*6*), appearing as bacteremia, ventriculitis, and brain abscess.

Microbiologic diagnosis of *Gordonia* spp. remains difficult. Biochemical profiles can lead to incorrect identification of isolates as *Rhodococcus* spp. (*2*,*4*–*6*,*9*) and sometimes *Corynebacterium* spp. (*3*) or *Nocardia* spp. (*6*). Identification at the genus and species levels is presently obtained by 16S rRNA sequence comparisons.

No recommendation for antimicrobial drug susceptibility testing has been unanimously approved, but these microorganisms seem to be susceptible to many antimicrobial drugs (*6*). Previous studies suggest a combination of penicillins and aminoglycosides as a suitable therapy for *Gordonia*-related bacteremia (*3*). Carbapenem or fluoroquinolone in combination with an aminoglycoside can also be used (*6*). Antimicrobial drug susceptibility is similar to that of *Rhodococcus* spp., for which *Gordonia* spp. are usually incorrectly identified. However, although vancomycin is often used to treat *Rhodococcus* spp. infections, in a previous study 11% of *Gordonia* spp. isolates were resistant (*6*). Treatment must therefore be evaluated specifically for each patient.

Gordonia spp. are environmental bacteria whose implication in human disease seems to be increasing. Phenotypic identification of bacteria included in this genus is difficult, and they are often poorly identified as *Rhodococcus* spp. or *Corynebacterium* spp. Molecular identification of *Gordonia* spp. by using 16S rRNA gene sequence comparison enables their characterization in human disease because the method is more accurate. The fact that these bacteria are often associated with medical devices highlights their role as nosocomial agents. Gram-positive bacilli must, therefore, not be systematically considered as contaminants, especially if associated with medical devices, and should be thoroughly identified by molecular methods in addition to biochemical tests.

Acknowledgment

We are grateful to Elena Rydkina for revising the English of the manuscript.

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DOI: 10.3201/eid1509.080903

References

1. Tsukamura M. Proposal of a new genus, *Gordona*, for slightly acid-fast organisms occurring in sputa of patients with pulmonary disease and in soil. J Gen Microbiol. 1971;68:15–24.

2. Lesens O, Hansmann Y, Riegel P, Heller R, Benaissa-Djellouli M, Martinot M, et al. Bacteremia and endocarditis caused by a *Gordonia* species in a patient with a central venous catheter. Emerg Infect Dis. 2000;6:382–5.
3. Verma P, Brown JM, Nunez VH, Morey RE, Steigerwalt AG, Pellegrini GJ, et al. Native valve endocarditis due to *Gordonia polyisoprenivorans*: case report and review of literature of bloodstream infections caused by *Gordonia* species. J Clin Microbiol. 2006;44:1905–8. DOI: 10.1128/JCM.44.5.1905-1908.2006
4. Sng LH, Koh TH, Toney SR, Floyd M, Butler WR, Tan BH. Bacteremia caused by *Gordonia bronchialis* in a patient with sequestrated lung. J Clin Microbiol. 2004;42:2870–1. DOI: 10.1128/JCM.42.6.2870-2871.2004
5. Gil-Sande E, Brun-Otero M, Campo-Cerecedo F, Esteban E, Aguilar L, García-de-Lomas J. Etiological misidentification by routine biochemical tests of bacteremia caused by *Gordonia terrae* infection in the course of an episode of acute cholecystitis. J Clin Microbiol. 2006;44:2645–7. DOI: 10.1128/JCM.00444-06
6. Blaschke AJ, Bender J, Byington CL, Korgenski K, Daly J, Petti CA, et al. *Gordonia* species: emerging pathogens in pediatric patients that are identified by 16S ribosomal RNA gene sequencing. Clin Infect Dis. 2007;45:483–6. DOI: 10.1086/520018
7. Werno AM, Anderson TP, Chambers ST, Laird HM, Murdoch DR. Recurrent breast abscess caused by *Gordonia bronchialis* in an immunocompetent patient. J Clin Microbiol. 2005;43:3009–10. DOI: 10.1128/JCM.43.6.3009-3010.2005
8. Iida S, Taniguchi H, Kageyama A, Yazawa K, Chibana H, Murata S, et al. *Gordonia otitidis* sp. nov., isolated from a patient with external otitis. Int J Syst Evol Microbiol. 2005;55:1871–6. DOI: 10.1099/ijs.0.63282-0
9. Blanc V, Dalle M, Markarian A, Debunne MV, Duplay E, Rodriguez-Nava V, et al. *Gordonia terrae*: a difficult-to-diagnose emerging pathogen? J Clin Microbiol. 2007;45:1076–7. DOI: 10.1128/JCM.02394-06
10. Jannat-Khah DP, Halsey ES, Lasker BA, Steigerwalt AG, Hinrikson HP, Brown JM. *Gordonia araii* infection associated with an orthopedic device and review of the literature on medical device-associated *Gordonia* infections. J Clin Microbiol. 2009;47:499–502. DOI: 10.1128/JCM.01504-08

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Cross-reactive Antibodies against Avian Influenza Virus A (H5N1)

To the Editor: Intravenous immunoglobulin (IVIg) is used to treat bacterial and viral infections in patients with primary immunodeficiency disease and those with autoimmune and inflammatory disorders (*1*). IVIg contains pooled IgG from >1,000 blood donors and antibodies against various common human pathogens, including influenza virus A.

We tested the efficacy of commercial preparations of IVIg (50 mg/mL of highly purified immunoglobulin) against homosubtypic influenza viruses A (H1N1 and H3N2) and their cross-reactivity against avian influenza virus A (H5N1). IVIg preparations (Octagam; Octapharma, Vienna, Austria and Flebogamma; Instituto Grifols, Barcelona, Spain) had hemagglutination inhibition (HI) titers against subtypes H1N1 and H3N2 ranging from 20 to 40. Human Immunoglobulin, pH 4.0, (Harbin Sequel Bio-Engineering Pharmaceutical, Harbin, People's Republic of China) had lower HI titers against avian influenza viruses (10 for subtype H3N2 and <10 for subtype H1N1). As expected, we did not detect antibodies against hemagglutinin (HA) of subtype H5N1 (A/open-billed/stork/Nahkonsawan/BBD0104F/2004) in any of the IVIg preparations (HI titer <10).

Human influenza subtype H1N1 shares the same neuraminidase (NA) subtype (human N1) as subtype H5N1 (avian N1). We therefore tested whether IVIg preparations would react and inhibit NA activity of human and avian influenza viruses by using a neuraminidase inhibition (NI) assay (*2*). NI titer was defined as the reciprocal of the highest dilution that gave 50% reduction compared with that of the virus control.

All 3 IVIg preparations inhibited NA activity of human N1 (NI titer against subtype H1N1 range 258–986) and human N2 (NI titer against subtype H3N2 range 1,309–3,274). Enzyme activity of avian N1 (7:1 reassortant; PR8 + NA [A/Vietnam/DT-0361/2005 H5N1]) was inhibited by all IVIg preparations (NI titer range 143–231). These findings support the recent observation of neutralizing antibodies against human N1 in human serum, which could inhibit enzyme activity of avian N1 from subtype H5N1 (*3*,*4*). We also tested IVIg preparations against reverse genetics subtype H5N3 virus in which the N3 NA was derived from H2N3 virus (6:1:1 reassortant; 6 internal genes from PR8 + HA (A/Vietnam/DT-0361/05 H5N1) + NA (A/duck/Germany 1207 H2N3) and observed no effect (NI titer <10). The N3 subtype belongs to avian influenza NA. Thus, antibodies against NA in IVIg appear to be specific for those circulating human influenza viruses (human N1 and human N2).

Unlike HA and NA, virus matrix 2 ectodomain (M2e) is highly conserved. Its presence on the surface of the viral particle makes it a potential target of antibody response similar to that for HA and NA (*5*,*6*). We assessed reactivity of IVIg preparations against a consensus M2e peptide derived from human influenza viruses of H1, H2, and H3 subtypes (MSLLTEVETPIRNEWGCRCNDSSD) and those derived from A/Hong Kong/156/97 H5N1 (MSLLTEVETLTRNGWGCRCSDSSD and A/Thailand/ SP-83/2004 H5N1 (MSLLTEVETPTRNEWECRCSDSSD) by using ELISA (*7*). Antibody titer was defined as the reciprocal of the highest dilution that had an optical density of 0.5 at 414 nm in our assay.

Results showed considerable variation among IVIg preparations, caused by M2e peptides derived from different influenza viruses (titer range 88–23,614). Among the 3 preparations, Human Immunoglobulin, pH 4.0, IVIg showed the highest titers against

all M2e peptides (consensus, 9,639; H5N1 Hong Kong, 3,519; and H5N1 Thailand, 23,614). Variation of antibody titers against M2e in IVIGs may be geographically dependent. Unlike Octagam and Flebogamma, Human Immunoglobulin, pH 4.0, IVIg was likely derived from blood donors in China. Octagam and Immunoglobulin, pH 4.0, IVIg were more reactive with M2e of avian influenza virus (H5N1) (A/Thailand/SP-83/2004) than with other M2e peptides.

We measured the ability of IVIg preparations to inhibit influenza subtype H5N1 replication by using a plaque-reduction assay. Subtype H5N1 (A/open-billed stork/ Nakhonsawan/BBD0104F/2004) was maintained as described (*8*). MDCK cells were infected with virus and agar containing various concentrations of IVIg was layered on top of these cells and incubated for 2 days. Results are shown in the Figure. IVIG inhibited plaque formation in a dose-dependent manner. Although plaques of heterogeneous size were observed in infected plates without IVIg, larger plaques were preferentially neutralized with increasing concentrations of IVIg in the agar (Figure).

Premixing excess M2e peptide with IVIg to absorb M2e-specific antibodies had no effect on plaque formation, indicating that antibodies against M2e in IVIg preparations were not responsible for neutralization of influenza subtype H5N1. Antibodies against M2e may have a role in protection against subtype H5N1 by another mechanism.

Our data suggest that the neutralizing activity against influenza subtype H5N1 in all 3 IVIg preparations was likely contributed by cross-reactive antibodies against avian N1. IVIg has been reported to have antiinflammatory activity (*9*,*10*). The immune suppressive effect of IVIg may benefit patients by reducing the cytokine storm. These data suggest use of IVIg, especially preparations containing high neutralizing activity against subtype H5N1, as adjunctive treatment for infection with highly pathogenic avian influenza virus (H5N1).

This study was supported by grant Y1-AI-5026-01 from the National Institutes of Health, National Institute of Allergy and Infectious Diseases, International Research in Infectious Disease.

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DOI: 10.3201/eid1509.090471

References

1. McClelland DB, Yap PL. Clinical use of immunoglobulins. Clin Haematol. 1984;13:39–74.
2. Lambre CR, Terzidis H, Greffard A, Webster RG. Measurement of anti-influenza neuraminidase antibody using a peroxidase-linked lectin and microtitre plates coated with natural substrates. J Immunol Methods. 1990;135:49–57. DOI: 10.1016/0022-1759(90)90255-T
3. Sandbulte MR, Jimenez GS, Boon AC, Smith LR, Treanor JJ, Webby RJ. Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. PLoS Med. 2007;4:e59. DOI: 10.1371/journal.pmed.0040059

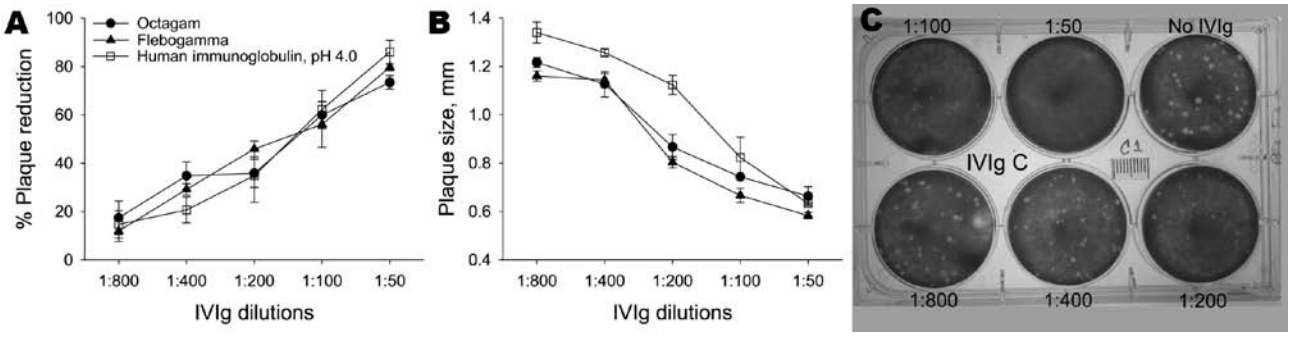

Figure. Neutralization of avian influenza virus A (H5N1) by intravenous immunoglobulin (IVIg) preparations measured by percentage reduction in plaque number (A) and plaque size (B). Monolayers of MDCK cells were infected with virus and overlaid with agar containing various concentrations of IVIg. After 2 days, plaques were detected by staining with crystal violet. Shown is a sample of viral plaques with agar overlay containing different dilutions (1:50–1:800) of Human Immunoglobulin, pH 4.0, (Harbin Sequel Bio-Engineering Pharmaceutical, Harbin, People's Republic of China) IVIg (C). Data are mean ± SE of 3 experiments. A color version of this figure is available online (www.cdc.gov/EID/content/15/9/1537-F.htm).

4. Lynch GW, Selleck PW, Axell A-M, Downton T, Kapitza NM, Boehm I, et al. Cross-reactive anti-avian H5N1 influenza neutralizing antibodies in a normal 'exposure-naive' Australian blood donor population. The Open Immunology Journal. 2008;1:13–9. DOI: 10.2174/1874226200801010013
5. Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med. 1999;5:1157–63. DOI: 10.1038/13484
6. Zharikova D, Mozdzanowska K, Feng J, Zhang M, Gerhard W. Influenza type A virus escape mutants emerge in vivo in the presence of antibodies to the ectodomain of matrix protein 2. J Virol. 2005;79:6644–54. DOI: 10.1128/JVI.79.11.6644-6654.2005
7. Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, Ye Z, et al. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg Infect Dis. 2007;13:426–35.
8. Thitithanyanont A, Engering A, Ekchariyawat P, Wiboon-ut S, Limsalakpetch A, Yongvanitchit K, et al. High susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-alpha and TLR ligands. J Immunol. 2007;179:5220–7.
9. Ephrem A, Misra N, Hassan G, Dasgupta S, Delignat S, Van Huyen JP, et al. Immunomodulation of autoimmune and inflammatory diseases with intravenous immunoglobulin. Clin Exp Med. 2005;5:135–40. DOI: 10.1007/s10238-005-0079-y
10. Ephrem A, Chamat S, Miquel C, Fisson S, Mouthon L, Caligiuri G, et al. Expansion of CD4+CD25+ regulatory T cells by intravenous immunoglobulin: a critical factor in controlling experimental autoimmune encephalomyelitis. Blood. 2008;111:715–22. DOI: 10.1182/blood-2007-03-079947

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The Last Taboo: Opening the Door on the Global Sanitation Crisis

Maggie Black and Ben Fawcett

Earthscan Publications Ltd, London, UK, and Sterling, VA, USA, 2008

ISBN: 978-1-84407-544-7
Pages: 224; Price: US $38.95

Toilets that Make Compost

Peter Morgan

Practical Action Publishing, Stockholm, Sweden, 2008

ISBN: 978-1-85339-674-8
Pages: 101; Price: US $39.95

The publication of each of these texts during 2008—declared the International Year of Sanitation by the United Nations General Assembly to draw attention to the 2.6 billion people without access to basic sanitation—is timely. Each seeks to provide practical information and guidance to increase the proportion of the population with access to sanitation.

Maggie Black is a writer who focuses on social development and sanitation, and Ben Fawcett is an environmental health engineer. In 7 chapters, they collaborated to provide a comprehensive overview of the history of the disposal of human waste, discuss how urbanization contributed to the sanitation crisis and the lack of response from local governments, describe programs that have developed with local support to address sanitation, detail the development and design of toilets, describe social marketing efforts to increase acceptance and use of toilets and their counterparts, detail the economic implications for sanitation programs, and outline a rationale for continuing to discuss a topic formerly been regarded as taboo.

Whereas Black and Fawcett devote ≈30 pages to a discussion of toilet design and models, Peter Morgan devotes his entire 100-page book to a hands-on discussion of the design of toilets that make compost. Using pictures and graphics, he describes toilets that could be easily reproduced by the novice at home and provides economically feasible designs for toilets that produce compost. Morgan suggests that the previously regarded "nuisance" human byproduct of toilet use can be used to fertilize vegetable gardens and other vegetation. Morgan proposes designs from simple to complex and details methods to reduce insects and odors, which are common annoyances associated with composting. In the book's introduction, he states, "It is possible to grow a tree directly in the filled toilet pit if it is planted in a layer of soil placed above the compost." He proceeds, "So the simple toilet can have many valuable uses, in addition to being a safe way to dispose of excreta."

Although the notion of eating a fruit or vegetable grown in excreta is unappealing to some, for most of the world's population, a solution to both the sanitation and waste-disposal conundrums results from building a toilet that has composting ability. Black and Fawcett's historical perspective on sanitation alludes to the potential environmental and economic implications of the benefit of such a strategy.

Each book is a worthwhile read for anyone interested in water and sanitation or in international health. Whereas Black and Fawcett's book is likely to appeal to a wider audience, Morgan's practical do-it-yourself approach is likely to especially interest persons involved in sanitation engineering. All 3 authors would have been delighted by the questions I received in airports, meetings, and transit while reading their respective books. The titles and topic remain an area of interest and fascination.

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DOI: 10.3201/eid1509.090668

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Abu'l Hasan, Nadir al-Zaman (1588–c.1635) Squirrels in a Plane Tree with Hunter Attempting to Climb the Tree (1605–08) Gouache on paper (22 cm × 36 cm) British Library, London, UK

Never Has There Been a Shade[1]

Polyxeni Potter

"On this date [in 1618] Abu'l Hasan the artist, who had been awarded the title Nadir al-Zaman [Wonder of the Age], presented a painting he had made …. Since it was worthy of praise, he was shown limitless favor. Without exaggeration, his work is perfect, and his depiction is a masterpiece of the age." These were the words of Emperor Jahangir of the Mughal Dynasty of India. "Abu'l Hasan's father," Jahangir continued, "was Aqa Reza of Herat [in western Afghanistan], who joined my service while I was still a prince. Abu'l Hasan therefore is a *khanazad* [born in the household, a second generation painter attached to the court]." His earliest known work, executed at age 12, a drawing after Albrecht Dürer's series the Apocalypse of St. John, already showed the promise of his mature work.

The Mughal Dynasty, known for its contributions in the political unification of India, also marked a golden age for the arts; particularly during the reign of Jahangir, when art intended to document the life and culture of the court flourished and a distinctive style developed known as Mughal painting. An opinionated collector and connoisseur, Jahangir was a literate and refined man. "I derive such enjoyment from painting and have such expertise in judging it," he wrote in his Memoirs, "that, even without the artist's name being mentioned, no work of past or present masters can be shown to me that I do not instantly recognize who did it."

Also a writer and naturalist, this aesthete emperor housed and recorded flora and fauna from near and afar. He took pity on elephants in winter and provided heated water for them to bathe in; he had shawls made for jackals to shield them against the cold. He encouraged detailed depictions of these and other animals, which his court painters produced prolifically, along with faithful copies of art prints, brought to India by missionaries.

During the 16th and 17th centuries, trade and the movement of humans, animals, and plants increased around the globe. Italian Renaissance reached the Mughal court, and the representational realism of the court, shaped by Hindu, Persian, and Chinese influences, found its way into European painting as Dutch and other artists visited India. Rembrandt collected Mughal works, while artists of the court

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DOI: 10.3201/eid1509.000000

[1] *Ombra mai fu* aria from George Frideric Handel's opera Xerxes.

copied Dürer and English painters Nicholas Hilliard and Isaac Oliver. Still, Mughal painters treaded their own path, resisting such preoccupations as perspective and the use of chiaroscuro to create the illusion of depth. Instead, they painted flat patterns, which they burnished for a jewel-like effect.

Despite Jahangir's far-sighted insistence that paintings be cataloged, dated, and even signed, little besides names is known about the artists who served in his and other courts or about their lives and status in society. Wages were roughly equivalent to those of soldiers, though bonuses were given for outstanding work, and artists traveled with the court to wars and hunts. Not many of Abu'l Hasan's paintings have survived, though the few that have show a variety of subjects, among them scenes of everyday life. His portraits and religious paintings were displayed in public places and were preserved in albums made for the emperor.

Squirrels in a Plane Tree, on this month's cover, the most famous painting associated with Abu'l Hasan, shows a naturalism that must have followed direct observation. And because the common red squirrels (*Sciurus vulgaris*) in it are European natives not found in India, the artist must have observed them either in Jahangir's zoo or abroad during one of the emperor's travels.

Backdrop for the gamboling creatures is the iconic plane tree (*Platanus orientalis*), a fixture of the Indian countryside and a royal tree to the Mughals. This was the Tree of Hippocrates, under which the sage physician taught medicine in Kos. With a range from Iberia to the Himalaya, it is known as chenar in Persia. Xerxes fell in love with it, his sentiment immortalized in the namesake opera by George Frideric Handel: "Never was there made/a shade of a plant/dear and loving,/or more gentle." Iqbal, poet of the East, traced the warmth of Kashmiri soil to the "blaze of chinars it nurses in its bosom."

Abu'l Hasan captured the five-lobed leaves, whose horizontal orientation accounts for the legendary shade of the tree. These leaves, which Kasmiris dry and turn into charcoal to fuel makeshift heaters (*kangris*) carried under their tunics in winter, change from deep green to bright red in the fall, their beauty continuing to ignite the artistic imagination.

Rightfully at center stage, Abu'l Hasan's plane tree is rooted in the bottom edge, while its canopy brushes the top of the composition. A couple of adult squirrels rein in nearly a dozen young, who cluster playfully in and around the trunk. Birds fly by, perch on the branches, or pick at the greenery on the ground, while goats frolic in the glade. Near the base, a hunter begins to climb toward the furry creatures. Like the squirrels, he does not have native features but seems extracted from some European painting of the period. The squirrels are disproportionately large; the local landscape dwarfed and compressed awkwardly around the exotic central theme.

This composition, too large to have been illustration for a manuscript, was probably derived from more than one source, its topical meaning now unknown. Some have surmised an allegorical connotation, one perhaps alluding to the adversarial relationship between humans and nature. But current concerns involving human–animal interaction and its many foibles invite even more tangible interpretations.

Abu'l Hasan's bucolic tree teeming with small mammals and birds and surrounded by wildlife seems pertinent to a topic in this issue of Emerging Infectious Diseases: Kyasanur Forest disease and the namesake mammalian tick-borne virus, enzootic to limited geographic areas of India's Karnataka State. The virus is transmitted by ticks between ground birds and small mammals. The recently discovered common ancestry of the Indian and Saudi Arabian Kyasanur Forest disease viruses, despite their large geographic separation, indicates long-range movement of virus, possibly by birds.

While no one knows why Abu'l Hasan's hapless hunter was climbing the plane, any modern viewer can surmise the futility of his bare-handed endeavor against acrobatic rodents and birds. Not to mention that, unbeknownst to him, along with other hunters of tree-dwelling creatures, he is at great risk for virus infection. Still, his predicament pales beside that of virus hunters, who know as in the case of Kyasanur Forest disease virus, that obscured by unrecognized disease or cryptic enzootic cycles, elusive viruses may exist in other geographic areas and ecologic niches.

Bibliography

1. Archer M, Archer WG. Indian painting for the British, 1770–1880. Oxford (UK): Oxford University Press; 1955.
2. Goswamy BN, Smith C. Domains of wonder: selected masterworks of Indian painting. Frome, Somerset (UK): Butler and Tanner, Ltd; 2005.
3. India office select materials [cited 2009 Jun 30]. Available from http://www.bl.uk/catalogues/indiaofficeselect/OIOCEnqFull.asp?ItemID=37&RecNo=1&intPDSearchNo=783009
4. Mehla R, Kumar SRP, Yadav P, Barde PV, Yergolkar PN, Erickson BR, et al. Recent ancestry of Kyasanur Forest disease virus. Emerg Infect Dis. 2009;15:1431–7.
5. The Mughals and the arts [cited 2009 Jun 23]. Available from http://www.coumbia.edu/itc/mealac/pritchett/00islamlinks/ikram/part2_18.html
6. Rogers A, Beveridge H. translators. Memoirs of Jahangir. London; 1909. Seth M. Indian painting. New York: Abrams; 2006.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Drug Prophylaxis to Prevent Influenza in Nursing Homes

Nontuberculous Mycobacteria Infections and Anti–Tumor Necrosis Factor–α

Nontuberculous Mycobacteria–associated Lung Disease, United States

Influenza Surveillance System, Beijing, China

Absence of Airborne Transmission during Pandemic (H1N1) 2009 Outbreak, China

Community-associated Methicillin-Resistant *Staphylococcus aureus*, Iowa, USA

Genetic Analysis of *Mycobacterium tuberculosis* Strains

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Clade 1 Influenza A Viruses (H5N1), South Indochina Peninsula

Escherichia coli as Reservoir for Macrolide Resistance Genes

Tick-borne Encephalitis from Eating Goat Cheese, Austria

West Nile Antibodies in Resident Wild Birds, Morocco

Rabies in Foxes, Aegean Region, Turkey

Complete list of articles in the October issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

October 29–November 1, 2009
47th Annual Meeting of IDSA and HIVMA
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http://www.idsociety.org/Content.aspx?id=12006

November 7–11, 2009
American Public Health Association 137th Annual Meeting and Exposition
Philadelphia, PA, USA
http://www.apha.org/meetings

November 18–22, 2009
American Society of Tropical Medicine and Hygiene 58th Annual Meeting
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December 4–6, 2009
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2010

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Berlin, Germany
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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

Extrapulmonary Infections Associated with Nontuberculous Mycobacteria in Immunocompetent Persons

CME Questions

1. Which of the following statements about nontuberculous mycobacterial (NTM) lymphadenitis is most accurate?

A. It is the most common NTM disease in children
B. The most frequently isolated organism is *Mycobacterium haemophilum*
C. NTM adenitis almost always presents with the sudden onset of severe illness
D. Chemotherapy is more effective than surgical excision for uncomplicated NTM lymphadenitis

2. Which of the following statements about osteoarticular infections with NTM is most accurate?

A. NTM are usually spread through the blood to the bones and joints
B. The ankle is the most common anatomic location of NTM tenosynovitis
C. The clinical presentation for NTM and tuberculous osteoarticular infections is very similar
D. Chemotherapy is unnecessary when surgical debridement is used to treat osteoarticular infections with NTM

3. Which of the following treatments is appropriately matched with its NTM skin or soft tissue infection?

A. *Mycobacterium marinum*: Doxycycline monotherapy is acceptable for severe infection
B. *Mycobacterium ulcerans*: Clarithromycin is the treatment of choice
C. *M. ulcerans*: Minocycline is the treatment of choice
D. M. avium complex: Treatment usually consists of 3 antibiotics for 6–12 months

4. Which of the following statements about rapidly growing mycobacteria (RGM) is most accurate?

A. They respond to sterilization with formaldehyde solutions only
B. They include organisms, such as *Mycobacterium fortuitum* and *Mycobacterium chelonae*
C. Symptoms of RGM always occur within 4 weeks of exposure
D. The usual duration of antibiotic therapy for infection with RGM is 1–2 months

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 www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- ★ New infections resulting from changes or evolution of existing organisms.
- ★ Known infections spreading to new geographic areas or populations.
- ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
   - ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - ★ Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - ★ Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - ★ Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

August 2009

Rabies

Courtesy of the Honolulu Academy of Arts, Hawai, USA Gift of James A. Michener, 1975 (16,576)

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.