EMERGING INFECTIOUS DISEASES®

May 2014

Infectious Diseases and Cancer

Arcimboldo, Flower petals on mistletoe mask, 14" x 13" x 12", 2009 (http://courageunmasked.org/)

EMERGING INFECTIOUS DISEASES®

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

∞ Emerging Infectious Diseases is printed on acid-free paper that meets the requirements of ANSI/NISO 239.48-1992 (Permanence of Paper)

EMERGING INFECTIOUS DISEASES

May 2014

On the Cover

B.J. Adams

***With Apologies to Arcimboldo.* Flower petals on radiation mask. 14″ x 13″ x 12″, 2009.**

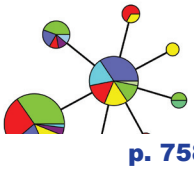
http://courageunmasked.org/

About the Cover p. 920

Perspective

Synopsis

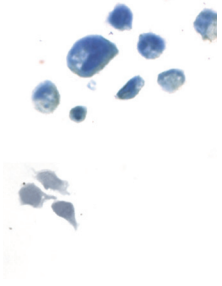
Medscape EDUCATION ACTIVITY



p. 758

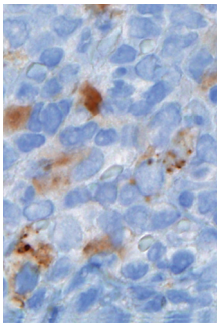
Research

p. 779



EMERGING INFECTIOUS DISEASES
May 2014

Dispatches



p. 834

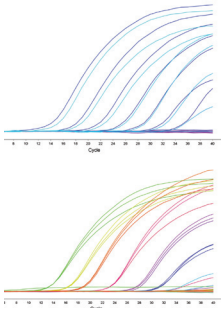
p. 839

Photo Quiz

EMERGING INFECTIOUS DISEASES

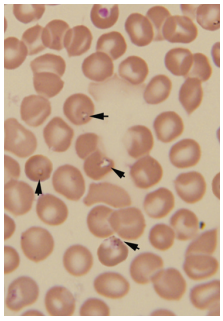
May 2014

Letters



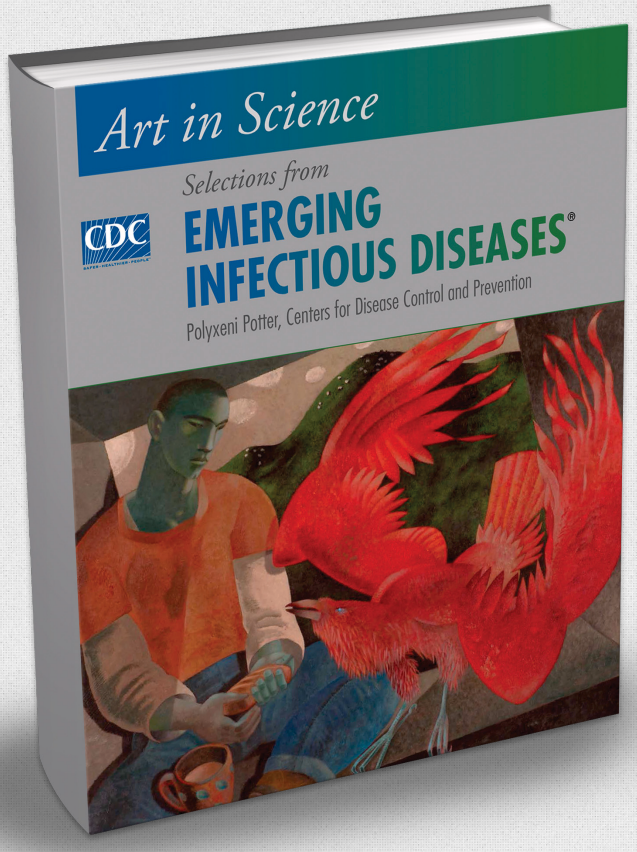
p. 848

p. 896



About the Cover

Etymologia



NOW AVAILABLE

Art in Science: **Selections from EMERGING INFECTIOUS DISEASES**

[Hardcover]

The journal's highly popular fine-art covers are contextualized with essays that address how the featured art relates to science, and to us all.

Available at http://bit.ly/16sJI6R Use promo code 32327 for an additional 20% off

This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Bat Flight and Zoonotic Viruses

Thomas J. O'Shea, Paul M. Cryan, Andrew A. Cunningham, Anthony R. Fooks, David T.S. Hayman, Angela D. Luis, Alison J. Peel, Raina K. Plowright, and James L.N. Wood

Bats are sources of high viral diversity and high-profile zoonotic viruses worldwide. Although apparently not pathogenic in their reservoir hosts, some viruses from bats severely affect other mammals, including humans. Examples include severe acute respiratory syndrome coronaviruses, Ebola and Marburg viruses, and Nipah and Hendra viruses. Factors underlying high viral diversity in bats are the subject of speculation. We hypothesize that flight, a factor common to all bats but to no other mammals, provides an intensive selective force for coexistence with viral parasites through a daily cycle that elevates metabolism and body temperature analogous to the febrile response in other mammals. On an evolutionary scale, this host–virus interaction might have resulted in the large diversity of zoonotic viruses in bats, possibly through bat viruses adapting to be more tolerant of the fever response and less virulent to their natural hosts.

Bats are a major source of zoonotic viruses worldwide (*1*–*3*). Molecular studies have demonstrated that bats are natural host reservoirs for several recently emerged high-profile zoonotic viruses, including sudden acute respiratory syndrome–like coronaviruses (*4*); Ebola and Marburg hemorrhagic fever filoviruses (*5*,*6*); rabies and rabies-related lyssaviruses; and many paramyxoviruses, including rubulaviruses and Nipah and Hendra viruses (*7*–*9*). Identification of a diverse range of bat paramyxoviruses, including those conspecific with human mumps virus, and phylogenetic reconstruction of host associations suggests numerous host switches of paramyxoviruses from bats to other mammals and birds (*9*). Bats infected with these viruses seem to show no overt signs of disease (*10*,*11*) and, in some cases, appear to be persistently infected (*12*). In the aggregate, zoonotic viruses in >15 virus families have been identified in at least 200 species in 12 bat families around the world (*3*,*9*,*11*). In a recent comparative analysis, Luis et al. (*3*) showed bats to be more likely to be infected with more zoonotic viruses per host species than were rodents, thus adding weight to the suggestion that bats might in some way be unique as sources of emerging zoonoses. In areas where these viruses have been studied, some viral groups (e.g., coronaviruses, astroviruses, paramyxoviruses) have much higher viral diversity and prevalence in bats than in sympatric species of rodents (*9*,*11*).

Although bats serve as reservoir hosts with great viral diversity, little evidence exists for corresponding death or illness of bats from viruses (other than lyssaviruses) that have spread into humans and domesticated mammals with high virulence (*9*,*10*). This statement also can be true for some viruses from other groups of mammals that serve as reservoirs of viruses, particularly rodents. However, low virulence coupled with high diversity has led to the suggestion that bats might have evolved mechanisms to control viral replication more effectively than have most other mammals (*10*,*13*) and that some attribute common to all bats (a very widely distributed and diverse group) also might explain the apparent low virulence of viral infections in these animals (*1*,*2*). We hypothesize that the increased metabolism and higher body temperatures of bats during flight might serve as an evolutionary adjuvant to their immune systems, providing a powerful selective force against virulence and promoting the diversity of viruses that infect bat populations. Perhaps counter-intuitively, this would enable bats to tolerate a greater diversity of viruses that have a high potential for virulence when transmitted to other mammals. The hypothesis also might help explain why co-evolved bat viruses cause high pathogenicity when they spill over into other mammals because the bat-derived viruses might survive well under both febrile and cooler conditions.

Author affiliations: US Geological Survey, Fort Collins, Colorado, USA (T.J. O'Shea, P.M. Cryan); Zoological Society of London, London, UK (A.A. Cunningham); Animal Health and Veterinary Laboratories Agency—Weybridge, Weybridge, UK (A.R. Fooks); National Consortium for Zoonosis Research, South Wirral, UK (A.R. Fooks); Colorado State University, Fort Collins, Colorado, USA (D.T.S. Hayman, A.D. Luis); University of Florida, Gainesville, Florida, USA (D.T.S. Hayman); Fogarty International Center of the National Institutes of Health, Bethesda, Maryland, USA (A.D. Luis); University of Cambridge, Cambridge, UK (A.J. Peel, J.L.N. Wood); Griffith University, Brisbane, Queensland, Australia (A.J. Peel); and Pennsylvania State University, University Park, Pennsylvania, USA (R.K. Plowright)

DOI: http://dx.doi.org/10.3201/eid2005.130539

Are Bats Special As Viral Hosts?

Three recent reports provide especially noteworthy background for this perspective. Luis et al. (*3*) performed a comparative analysis of viruses in bats and rodents (the most speciose group of mammals) and associated ecologic factors. The analysis indicated that bats are indeed special in hosting more viruses per species than rodents, despite twice as many rodent species in the world, and that certain ecologic factors are associated with the hosting of more viruses by bats (*3*). Through an extensive genomic analysis of 2 divergent species of bats, Zhang et al. (*13*) found that flight evolved in tandem with concomitant genetic changes to their innate immune systems. These changes were consistent with the need for DNA damage repair because of high metabolic rates during flight (*13*). Baker et al. (*10*) reviewed antiviral immune responses in bats and suggested the possibility that bats might be able to control viral replication through innate immunity. They summarized research indicating that bats have immune elements found in most other mammals, including pattern recognition receptors and multiple interferons, and show complement activity (*10*). Many standard elements of the adaptive immune system also have been described in bats, including multiple immunoglobulins, antibody responses, interleukins and other cytokines, and cell-mediated T-cell responses (although investigations of the latter have been limited [*10*]). However, genes that code for some immune elements are notably lacking in bats and indicate permanent change to the bat immune system (*13*).

Fever, Flight, and Metabolic Rate

Fever in mammals is accompanied by an initially high metabolic rate associated with beneficial immune responses (*14*,*15*). During flight, bats exhibit a high increase in metabolic rate over the resting metabolic rate of normeothermic, otherwise active bats. This increase is estimated to be 15–16-fold (*16*), in comparison with the 7-fold increase in metabolic rates of rodents running to exhaustion (*17*) or the 2-fold increase in metabolic rates of most flying birds (*18*). Strains of laboratory mice that are inbred for higher metabolic rates show stronger immune responses to immune challenge (keyhole limpet hemocyanin antigen) with stronger antigen-specific IgM production than strains bred for lower metabolic rates; leukocyte counts and mass of lymphatic organs that are the sources of immune cells involved in antigen recognition and elimination also are elevated in the strains with higher metabolic rates (*19*). The metabolic cost of raising an immune response to experimental stimulation typically results in a general increase of ≈10%–30% of resting metabolic rates in a variety of nonvolant small mammals (*20*,*21*). When a bat is confronted by a viral antigen, the proportional increase in metabolism for raising an immune response may be trivial compared with the very large increase in the metabolic costs of flight (the proportional increase for flight may even be greater, given the wider metabolic scope of many species of bats that undergo shallow daily torpor). Thus, we hypothesize that the higher metabolic rates during flight in bats may enhance, facilitate, or perhaps subsidize any inherent cost of raising metabolism to activate an immune response. The daily cyclical nature of raised metabolism during flight also might enable some viruses to persist within the bats and perhaps become resistant to this part of the innate immune response.

Bat Flight and Elevated Body Temperatures

Canale and Henry (*22*) stated, "The heat of fever forestalls pathogen replication and increases the efficiency of the immune responses. Such body warming is associated with shortened disease duration and improved survival in most animals." Although fever has been associated with improved recovery, very little is known about mechanisms, including whether the impact involves thresholds or average rates of immune response. During fever, mammalian core body temperatures can vary but typically are 38°C–41°C (*14*). The high metabolic demands of bat flight result in core body temperatures that commonly reach the ranges of core temperatures typical of fever. High body temperatures during flight have been demonstrated in multiple species of bats within several families (Table 1), and such high body temperature ranges increase the rate of multiple immune responses in mammals, including components of the innate and adaptive immune systems (Table 2). Daily high body temperatures thus might arm bats against some pathogens during the early stages of infection. An exception to the daily high body temperatures during flight occurs during hibernation in temperate zones: although bats rouse from hibernation multiple times each winter (*35*), replication of most mammalian adapted pathogens is expected to be markedly reduced by the lower core body temperatures of hibernation.

A Speculative Hypothesis

If the elevated metabolic rates and body temperatures accompanying flight facilitate activation of the immune system of bats on a daily cycle, then flight could be the ultimate explanatory variable for the evolution of viral infections without overt signs of illness in bats. Zhang et al. (*13*) showed that the evolution of flight in bats has been accompanied by genetic changes to their immune systems to accommodate high metabolic rates. Theoretical models of the evolution of parasite virulence show that intermediate levels of virulence are a typical result of the trade-off between the opposing selective forces of host death and parasite transmission (*36*). However, heightened host adaptive immune responses that might be facilitated in bats during flight also can result in harmful immunopathologic changes and disease. (A nonviral example of such harmful

Table 1. Examples of elevated core body temperature in flying bats*

Bat species (family)	Core temperature during flight, °C	Source
Eidolon helvum (Pteropidae)	36.9–40.8	(*23*)
Hypsignathus monstrosus (Pteropidae)	37.2–40.0	(*23*)
Rousettus aegyptiacus (Pteropidae)	38.2–41.2	(*23*)
Rhinolophus ferrumequinum (Rhinolophidae)	41†	(*24*)
Miniopterus sp. (Miniopteridae)	41.1 ± 0.45	(*25*)
Phyllostomus hastatus (Phyllostomidae)	41.2–42.1	(*17*)
Carollia perspicillata (Phyllostomidae)	40.2 ± 0.8	(*26*)
Artibeus lituratus (Phyllostomidae)	c. 41.2 ± 1	(*27*)
Sturnira lilium (Phyllostomidae)	c. 40.5 ± 0.3	(*27*)
Noctilio albiventris (Noctilionidae)	35.5–40.6	(*28*)
Myotis yumanensis (Vespertilionidae)	40.0–40.8	(*29*)
Eptesicus fuscus (Vespertilionidae)	41.3 ± 2.1†, 37–39.5‡	(*30*)
Mops condylurus (Molossidae)	40.5 ± 1.1	(*31*)
Tadarida brasiliensis (Molossidae)	35–42	(*32*)
Eumops perotis (Molossidae)	37.8–39.3	(*33*)
Myotis volans (Vespertilionidae)	37.4	(*34*)
Myotis evotis (Vespertilionidae)	38.3	(*34*)
Myotis californicus (Vespertilionidae)	38.4	(*34*)
Parastrellus hesperus (Vespertilionidae)	38.8	(*34*)
Eptesicus fuscus (Vespertilionidae)	41.0	(*34*)
Lasiurus cinereus (Vespertilionidae)	40.2	(*34*)
Antrozous pallidus (Vespertilionidae)	40.6	(*34*)
Tadarida brasiliensis (Molossidae)	38.0	(*34*)

*Data available from original sources are given as ranges or means ±1 SD.
†Skin temperature.
‡Body temperature.

immunopathology in bats seems to occur during infection by the fungal pathogen causing white-nose syndrome (*Pseudogymnoascus destructans*) when hibernating bats resume flight [*37*]). In systems in which disease organisms cause major immunopathologic changes, theoretical analyses suggest that natural selection can favor decreased virulence and incomplete clearance of parasites (*36*,*38*). Through heightened immunopathologic responses, flight might have been a potent selective factor for the reduced virulence to the natural hosts seen in the pool of emerging viruses recently discovered in bats. It also is notable that there are few reports of mass deaths from diseases in bats (except for the novel white-nose syndrome fungus), despite reports of bat die-offs due largely to other causes that have appeared in the literature over the years (e.g., review in *39*). The evolution of flight in conjunction with the bat's speculated heightened potential for immune vigilance might have predisposed bats to be reservoir hosts to a preponderance of viruses that now lack major effects on bats as the natural host populations but that can emerge into populations of humans and domesticated mammals with greater virulence.

Consideration of the role of torpor is also germane to our hypothesis. As noted by Luis et al. (*3*), "more research is needed to determine the relationship between torpor, host competence as related to within-host viral persistence and population viral perpetuation processes." Viral replication is dampened under the cooler host body temperatures that prevail during prolonged torpor, and hypothermia has been considered to be a host strategy that is adaptive against pathogens (*40*). Luis et al. (*3*) hypothesized that the negative correlation identified between the use of torpor and zoonotic viral richness may be due to lower contact rates, yet in bats it is also consistent with longer periods with no or lower flight activity and, consequently, lower frequency of the hypothesized heightened vigilance against invading viruses during the course of host–parasite co-evolution. On the other hand, reduced immune system activity during torpor may enable cold-adapted pathogens to persist (*40*), as in the case of the novel fungal pathogen causing white-nose syndrome. As a group, bats show a wide range of adaptations involving torpor that varies with latitude and phylogeny, ranging from prolonged deep winter hibernation through shallow daily torpor to year-round homeothermy. Viruses that have coevolved with bats under these conditions might have properties that can favor survival under a wider scope of temperatures, possibly facilitating spillover to novel hosts.

Testing the Hypothesis

In their review of bat immunology and viral diversity, Wang et al. (*11*) posed the question "Flight capability, longevity and innate immunity—are they linked?" and noted that "data in this field are so limited that it is … important to provoke original, speculative or even controversial ideas or theories in this important field of research." Our "flight-as-fever" hypothesis suggests 1 mechanism unique to bats that might be key in the flight capability–innate immunity linkage question raised by Wang et al. (*11*). Researchers interested in testing this hypothesis will find it challenging and demanding of creativity. Unfortunately, no prior studies have investigated the effect of the high

Table 2. Favorable innate and adaptive immune responses associated with the high body temperature of fever in mammals*

Enhanced neutrophil and monocyte motility and emigration
Enhanced phagocytosis and pinocytosis
Increased oxygen radical production by phagocytes
Increased interferon production
Increased antiviral, antitumor, or antiproliferative, and natural killer cell stimulating activities of interferon
Potentiated interferon-induced anti-anaphylaxis (anergy)
Enhanced natural killer complement activation
Enhanced expression of Fc receptors
Increased T-helper cell activation, expression, recruitment, and cytotoxic activity
Blocked T-suppressor cell activity
Increased antibody production
Enhanced tumor necrosis factor-α
Increased T-cell proliferative response to nonspecific mitogens, interleukin-1 and –2, and allogeneic lymphocytes
Increased killing of intracellular bacteria
Increased bactericidal effect of antimicrobial agents
Induced cytoprotective heat-shock proteins in host cells
Induced pathogen heat-shock proteins, which activate host defenses
Induced cytoprotective heat-shock proteins in host cells

*See reviews in (*14,15*).

body temperatures and metabolic rates associated with flight on host–virus interactions in bats.

However, we suggest that a variety of in vivo, in vitro, and in silico approaches be considered. For example, in vivo approaches could rely on the experimental techniques that were used decades ago in the pioneering studies of bat flight physiology (*16*,*17*). As in these prior physiologic studies, captive bats can be trained to fly in wind tunnels. Experiments could be designed to determine whether trained bats allowed to fly show heightened immune responses compared with when they were not flying. These experiments could begin with determining immune responses after exposure to harmless antigens and then progress to experiments involving exposure to viruses. In vitro studies could determine the comparative susceptibility of bat viruses and nonbat viruses grown in culture to altered and variable thermal regimes typical of the body temperatures of bats during flight, as well as bats in torpor. (In this regard, we note that most bat viruses that have been identified by using genetic techniques have not been isolated, described morphologically, or grown in culture; additional research using techniques of classical virology are certainly needed to improve the understanding of bat virology). Finally, in silico techniques of modeling and simulation would be helpful in understanding the likely co-evolution of bat viruses and their hosts when subject to daily fever-like thermal and metabolic regimes. Regimes of such frequency might accelerate the pace of co-evolution in the otherwise slowly evolving hosts, and favor the development of low pathogenicity in their much more rapidly evolving viruses.

Acknowledgments

We thank Clark Blatteis, Dave Blehert, Richard Bowen, Ed Clint, and Tony Schountz for reviewing earlier drafts of the manuscript.

This perspective was developed during discussions within the Small Mammals Working Group of the Research and Policy for Infectious Disease Dynamics (RAPIDD) program of the Science and Technology Directorate (US Department of Homeland Security) and the Fogarty International Center (National Institutes of Health). D.T.S.H. acknowledges funding from a David H. Smith Fellowship from the Cedar Tree Foundation and Society of Conservation Biology. A.D.L. acknowledges funding from a RAPIDD fellowship.

Dr O'Shea is scientist emeritus at the Fort Collins Science Center, US Geological Survey, Fort Collins, Colorado. His research interests include infectious disease ecology in bats and the ecology and conservation of bats and marine mammals.

References

1. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T. Bats: important reservoir hosts of emerging viruses. Clin Microbiol Rev. 2006;19:531–45. http://dx.doi.org/10.1128/CMR. 00017-06
2. Dobson AP. What links bats to emerging infectious diseases? Science. 2005;310:628–9. http://dx.doi.org/10.1126/science.1120872
3. Luis AD, Hayman DTS, O'Shea TJ, Cryan PM, Gilbert AT, Pulliam JRC, et al. A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special? Proc Royal Soc B Biol Sci. 2013;280:20122753. http://dx.doi.org/10.1098/rspb.2012.2753
4. Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, et al. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature. 2013;503:535–8. http://dx.doi.org/10.1038/nature12711
5. Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P, et al. Fruit bats as reservoirs of Ebola virus. Nature. 2005;438:575–6. http://dx.doi.org/10.1038/438575a
6. Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, Kemp A, et al. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. PLoS Pathog. 2009;5:e1000536. http://dx.doi.org/10.1371/journal.ppat.1000536
7. Halpin K, Young PL, Field HE, Mackenzie JS. Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. J Gen Virol. 2000;81:1927–32.

8. Chua KB, Koh CL, Hooi PS, Wee KF, Khong JH, Chua BH, et al. Isolation of Nipah virus from Malaysian Island flying foxes. Microbes Infect. 2002;4:145–51. http://dx.doi.org/10.1016/S1286-4579(01)01522-2
9. Drexler JF, Corman VM, Müller MA, Maganga GD, Vallo P, Binger T, et al. Bats host major mammalian paramyxoviruses. Nat Commun. 2012;3:796. http://dx.doi.org/10.1038/ncomms1796
10. Baker ML, Schountz T, Wang LF. Antiviral immune responses of bats: a review. Zoonoses Public Health. 2013;60:104–16. http://dx.doi.org/10.1111/j.1863-2378.2012.01528.x
11. Wang LF, Walker PJ, Poon LLM. Mass extinctions, biodiversity and mitochondrial function: are bats 'special' as reservoirs for emerging viruses? Curr Opin Virol. 2011;1:649–57. http://dx.doi.org/10.1016/j.coviro.2011.10.013
12. Sohayati AR, Hassan L, Sharifah SH, Lazarus K, Zaini CM, Epstein JH, et al. Evidence for Nipah virus recrudescence and serological patterns of captive *Pteropus vampyrus*. Epidemiol Infect. 2011;139:1570–9. http://dx.doi.org/10.1017/S0950268811000550
13. Zhang G, Cowled C, Shi Z, Huang Z, Bishop-Lilly KA, Fang X, et al. Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. Science. 2013;339:456–60. http://dx.doi.org/10.1126/science.1230835
14. Blatteis CM. Fever: pathological or physiological, injurious or beneficial? J Therm Biol. 2003;28:1–13. http://dx.doi.org/10.1016/S0306-4565(02)00034-7
15. Hasday JD, Fairchild KD, Shanholtz C. The role of fever in the infected host. Microbes Infect. 2000;2:1891–904. http://dx.doi.org/10.1016/S1286-4579(00)01337-X
16. Speakman JR, Thomas DW. Physiological ecology and energetics of bats. In: Kunz TH, Fenton MB, editors. Bat ecology. Chicago: University of Chicago Press; 2003. p. 430–90.
17. Thomas SP, Suthers RA. The physiology and energetics of bat flight. J Exp Biol. 1972;57:317–35.
18. Bundle MW, Hansen KS, Dial KP. Does the metabolic rate–flight speed relationship vary among geometrically similar birds of different mass? J Exp Biol. 2007;210:1075–83. http://dx.doi.org/10.1242/jeb.02727
19. Książek A, Konarzewski M. Effect of dietary restriction on immune response of laboratory mice divergently selected for basal metabolic rate. Physiol Biochem Zool. 2012;85:51–61. http://dx.doi.org/10.1086/663696
20. Cutrera AP, Zenuto RR, Luna F, Antenucci CD. Mounting a specific immune response increases energy expenditure of the subterranean rodent *Ctenomys talarum* (tuco-tuco): implications for intraspecific and interspecific variation in immunological traits. J Exp Biol. 2010;213:715–24. http://dx.doi.org/10.1242/jeb.037887
21. Martin LB, Weil ZM, Nelson RJ. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. Philos Trans R Soc Lond B Biol Sci. 2008;363:321–39. http://dx.doi.org/10.1098/rstb.2007.2142
22. Canale CI, Henry PY. Energetic costs of the immune response and torpor use in a primate. Funct Ecol. 2011;25:557–65. http://dx.doi.org/10.1111/j.1365-2435.2010.01815.x
23. Carpenter RE. Flight physiology of intermediate-sized fruit bats (Pteropodidae). J Exp Biol. 1986;120:79–103.
24. Burbank RC, Young JZ. Temperature changes and winter sleep of bats. J Physiol. 1934;82:459–67.
25. Morrison P. Body temperatures in some Australian mammals. I. Chiroptera. Biol Bull. 1959;116:484–97. http://dx.doi.org/10.2307/1538957
26. Voigt CC, Lewanzik D. Trapped in the darkness of the night: thermal and energetic constraints of daylight flight in bats. Proc Biol Sci. 2011;278:2311–7. http://dx.doi.org/10.1098/rspb.2010.2290
27. Morrison P, McNab BK. Temperature regulation in some Brazilian phyllostomid bats. Comp Biochem Physiol. 1967;21:207–21. http://dx.doi.org/10.1016/0010-406X(67)90130-2
28. Roverud RC, Chappell MA. Energetic and thermoregulatory aspects of clustering behavior in the neotropical bat *Noctilio albiventris*. Physiol Zool. 1991;64:1527–41.
29. Reeder WG, Cowles RB. Aspects of thermoregulation in bats. J Mammal. 1951;32:389–403. http://dx.doi.org/10.2307/1375787
30. Willis CKR, Brigham RM. Defining torpor in free-ranging bats: experimental evaluation of external temperature-sensitive radio-transmitters and the concept of active temperature. J Comp Physiol B. 2003;173:379–89. http://dx.doi.org/10.1007/s00360-003-0343-y
31. Bronner GN, Maloney SK, Buffenstein R. Survival tactics within thermally-challenging roosts: heat tolerance and cold sensitivity in the Angolan free-tailed bat, *Mops condylurus*. S Afr J Zool. 1999;34:1–10.
32. Herreid CF II. Temperature regulation and metabolism in Mexican freetail bats. Science. 1963;142:1573–4. http://dx.doi.org/10.1126/science.142.3599.1573
33. Leitner P. Body temperature, oxygen consumption, heart rate and shivering in the California mastiff bat, *Eumops perotis*. Comp Biochem Physiol. 1966;19:431–43. http://dx.doi.org/10.1016/0010-406X(66)90152-6
34. O'Farrell MJ, Bradley WG. Comparative thermal relationships of flight for some bats in southwestern United States. Comp Biochem Physiol A. 1977;58:223–7. http://dx.doi.org/10.1016/0300-9629(77)90230-4
35. Thomas DW. The physiological ecology of hibernation in vespertilionid bats. In: Racey PA, Swift, SM, editors. Ecology, evolution, and behaviour of bats. Oxford (UK): Clarendon Press; 1995. p. 233–44.
36. Long GH, Boots M. How can immunopathology shape the evolution of parasite virulence? Trends Parasitol. 2011;27:300–5. http://dx.doi.org/10.1016/j.pt.2011.03.012
37. Meteyer CU, Barber D, Mandl JN. Pathology in euthermic bats with white nose syndrome suggests a natural manifestation of immune reconstitution inflammatory syndrome. Virulence. 2012;3:583–8. http://dx.doi.org/10.4161/viru.22330
38. Graham AL, Allen JE, Read AF. Evolutionary causes and consequences of immunopathology. Annu Rev Ecol Evol Syst. 2005;36:373–97. http://dx.doi.org/10.1146/annurev.ecolsys.36.102003.152622
39. Clark DW Jr. Bats and environmental contaminants: a review. US Fish and Wildlife Service Special Scientific Report—Wildlife 1981; 235:1–27.
40. LeGrand EK, Alcock J. Turning up the heat: immune brinksmanship in the acute-phase response. Q Rev Biol. 2012;87:3–18. http://dx.doi.org/10.1086/663946

Address for correspondence: Thomas J. O'Shea, US Geological Survey, Fort Collins Science Center, 2150 Centre Ave, Bldg C, Fort Collins, CO 80526-8118, USA; email: osheat@usgs.gov

Outbreaks of *Kingella kingae* Infections in Daycare Facilities

Pablo Yagupsky

Medscape EDUCATION ACTIVITY

Medscape, LLC is pleased to provide online continuing medical education (CME) for this journal article, allowing clinicians the opportunity to earn CME credit.

This activity has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of Medscape, LLC and Emerging Infectious Diseases. Medscape, LLC is accredited by the ACCME to provide continuing medical education for physicians.

Medscape, LLC designates this Journal-based CME activity for a maximum of 1 *AMA PRA Category 1 Credit(s)*™. Physicians should claim only the credit commensurate with the extent of their participation in the activity.

All other clinicians completing this activity will be issued a certificate of participation. To participate in this journal CME activity: (1) review the learning objectives and author disclosures; (2) study the education content; (3) take the post-test with a 70% minimum passing score and complete the evaluation at **www.medscape.org/journal/eid**; (4) view/print certificate.

Release date: April 9, 2014; Expiration date: April 9, 2015

Learning Objectives

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

- Distinguish anatomic sites of invasive *Kingella kingae* infections
- Analyze the clinical presentation of invasive *K. kingae* infections
- Evaluate the diagnostic process for invasive *K. kingae* infections
- Assess the treatment of contacts of children with invasive *K. kingae* infections.

CME Editor

Jean Michaels Jones, Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: Jean Michaels Jones has disclosed no relevant financial relationships.*

CME Author

Charles P. Vega, MD, Associate Professor and Residency Director, Department of Family Medicine, University of California, Irvine. *Disclosure: Charles P. Vega, MD, has disclosed no relevant financial relationships.*

Author

Disclosure: ***Pablo Yagupsky, MD****, has disclosed no relevant financial relationships.*

During the past decade, transmission of the bacterium *Kingella kingae* has caused clusters of serious infections, including osteomyelitis, septic arthritis, bacteremia, endocarditis, and meningitis, among children in daycare centers in the United States, France, and Israel. These events have been characterized by high attack rates of disease and prevalence of the invasive strain among asymptomatic classmates of the respective index patients, suggesting that the causative organisms benefitted from enhanced colonization fitness, high transmissibility, and high virulence. After prophylactic antibacterial drugs were administered to close contacts of infected children, no further cases of disease were detected in the facilities, although test results showed that some children still carried the bacterium. Increased awareness of this public health problem and use of improved culture methods and sensitive nucleic acid amplification assays for detecting infected children and respiratory carriers are needed to identify and adequately investigate outbreaks of *K. kingae* disease.

Author affiliation: Soroka University Medical Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel

DOI: http://dx.doi.org/10.3201/eid2005.131633

During the past 3 decades, Western countries have reported a rising number of mothers entering the workforce and, consequently, a growing number of children receiving care outside the home (*1*). This trend has substantial public health consequences because the incidence of infectious diseases in general, and of those caused by respiratory pathogens in particular, has substantially increased among daycare center attendees (*1*,*2*). These organisms are usually spread within daycare centers by child-to-child transmission; they colonize the upper respiratory tract surfaces, from which they can disseminate to other attendees. From the upper airways, pathogens may invade adjacent structures such as the lungs, middle ear, or nasal sinuses, and may penetrate into the bloodstream, causing invasive diseases (*1*). Most bacterial pathogens responsible for such infections are enclosed by polysaccharide capsules that protect them from phagocytosis and complement-mediated killing, ensuring their persistence on the respiratory mucosae and survival in the bloodstream and deep body tissues (*3*). Maturation of the T-cell independent arm of the immune system in humans is delayed until the age of 2–4 years; thus, young children are prone to colonization and infection by encapsulated bacteria (*3*).

Besides the microorganisms' virulence and the hosts' age-related immunologic immaturity, many other factors contribute to the enhanced colonization, transmission, and illness rates observed among children in daycare centers, including the number of children present, the degree of crowding, efficacy of ventilation, time spent in day care, length of time from enrollment, frequency of enrollment, age group mixing, and occurrence of seasonal viral infections (*1*,*4*–*6*). Because of age stratification, child-care groups comprise attendees of approximately the same age who have similar degrees of immunologic immaturity and susceptibility to infectious agents. This epidemiologic setting substantially differs from that of large families in that the latter include children of different ages and therefore, at any given time, only a fraction of siblings belong to the age group at enhanced risk for bacterial colonization and invasion, which limits the chances to acquire and transmit the organism (*7*). In daycare centers, respiratory organisms spread easily through large droplet transmission among young children with poor hygienic habits who share toys contaminated with respiratory secretions or saliva. Under these circumstances, introduction of a virulent bacterium in a crowded daycare facility attended by immunologically naïve children may result in prompt dissemination of the organism and initiate outbreaks of disease such as those caused by pneumococci, *Haemophilus influenzae* type b, or *Neisseria meningitidis* (*1*,*2*,*6*).

Kingella kingae: An Emerging Pathogen in Young Children

Because of the improved culture methods (*8*,*9*) and sensitive nucleic acid amplification assays (NAAAs) (*10*–*14*) developed in recent years, *Kingella kingae*, a gram-negative coccobacillus of the *Neisseriaceae* family, is increasingly recognized as an invasive pathogen of early childhood. The organism is a frequent source of childhood bacteremia and the most common agent of skeletal system infections in children 6 months–3 years of age; it is also a cause of bacterial endocarditis in children and adults (*8*–*16*). Because of the fastidious nature of *K. kingae*, many illnesses caused by this bacterium are, probably, overlooked. Although most cases of invasive *K. kingae* infections are sporadic, clusters of invasive disease have been detected among attendees of daycare centers in Israel, Europe, and the United States.

Misdiagnosis

The bacteriologic identification of *K. kingae* relies on the following: typical Gram stain results, showing pairs or short chains of plump, gram-negative bacilli with tapered ends; β-hemolysis; pitting of the agar surface; failure to grow on MacConkey medium; weak oxidase activity and a negative catalase reaction; and, with rare exceptions, production of acid from glucose and maltose (*8*,*9*). However, *K. kingae* tends to retain crystal violet dye and, therefore, it may appear to be gram-positive, and laboratories unfamiliar with its cultural and staining features may misidentify the bacterium altogether or dismiss invasive isolates as culture contaminants. Identification of *K. kingae*, however, is not difficult, and many commercial instruments and technologies such as VITEK 2 (bioMérieux, Marcy-l'Etoile, France), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), or 16S rDNA gene sequencing, correctly identify the organism.

Characteristics and Manifestations

Recent studies have demonstrated the presence of a polysaccharide capsule on the surface of *K. kingae* that exhibits between-strains chemicals and, most probably, antigenic variation (*17*,*18*). These characteristics may assist *K. kingae* in evading the immune response by facilitating the successive colonization of the host by strains harboring different capsular types. The polysaccharide capsule probably enables mucosal colonization, the survival of the organism in the bloodstream, and invasion of deep body sites (*17*,*18*). In addition, all *K. kingae* strains produce and secrete a potent repeats-in-toxin (RTX) that exhibits a wide range of cytotoxic activity and is particularly deleterious to macrophage-like cells, leukocytes, and synovial cells and, to a lesser degree, to respiratory epithelial cells that improve the organism's chances of surviving in the host and of invading skeletal tissues (*12*,*19*).

Similar to meningococci, *K. kingae* is carried on the oropharyngeal epithelium (*20*,*21*), and the colonized mucosa is the portal of entry of the organism to the bloodstream from which it may disseminate to 3 areas for which the bacterium shows particular tropism: joints, bones, or the endocardium (*22*,*23*). Damage to the upper respiratory surfaces by previous or concurrent viral infections or stomatitis appears to facilitate bloodstream invasion by *K. kingae* (*16*).

K. kingae isolates show remarkable genomic diversity and, to date, 37 multilocus sequence typing (MLST) and 74 pulsed field gel electrophoresis (PFGE) clones have been identified (*24*–*26*). Carried *K. kingae* organisms differ in their invasive capabilities (*27*), and simultaneous carriage of ≥1 genotype is unusual (*28*). Whereas some strains, which are frequently isolated from healthy carriers, are seldom if ever detected in patients with clinical disease, others are rarely carried asymptomatically, but are responsible for a high proportion of invasive disease (*24*,*27*,*28*). However, a few strains appear to possess an optimal balance between transmissibility and invasiveness and are common among healthy carriers and among infected patients (*24*,*27*,*28*). A recent study has found that certain virulent *K. kingae* clones, characterized by a distinct combination of PFGE and MLST profiles, are substantially associated with bacteremia with no focal infection, skeletal system invasion, or endocarditis, which suggests biological specialization for invading tissues of specific hosts (*25*).

Most young children in whom an invasive *K. kingae* disease developed have been otherwise healthy. In contrast, children >4 years of age and adults who become infected frequently have underlying conditions such as congenital heart diseases, chronic renal failure, or a variety of primary immunodeficiencies (*16*).

The prevalence rate in healthy children during the second year of life ranges between 10% and 12% (*7*,*28*), which coincides with the peak attack rate of invasive infections (*16*). The colonization rate drops substantially in older children and adults (*7*,*28*,*29*). Pharyngeal carriage of *K. kingae* and occurrence of disease before a child is 6 months of age are exceptions, indicating that maternal immunity and limited social contact provide protection (*7*,*16*,*30*).

Diagnosis of *K. kingae* Infections

The clinical features of invasive *K. kingae* infections (other than endocarditis) are usually mild, and diagnosis requires a high level of suspicion by clinicians. Many patients with *K. kingae*–associated joint or bone disease are afebrile and blood leukocyte counts, C-reactive protein levels, and erythrocyte sedimentation rates are frequently normal (*16*,*31*,*32*). Although bacteremia with no focus is the second most frequent manifestation of invasive *K. kingae* infections, the condition is probably unsuspected and the diagnosis is likely missed in a large number of cases. The current guidelines for managing illness in young, febrile children with no apparent source of infection, which use body temperature and leukocyte count as criteria for obtaining blood cultures (*33*), are not sensitive enough for detecting occult *K. kingae* bacteremia because many infected children have a low-grade fever and may or may not have leukocytosis.

Detection of *K. kingae* infections by culture is highly dependent on the use of adequate laboratory techniques. The recovery of *K. kingae* from synovial fluid and bone exudates seeded onto routine solid culture media is suboptimal (*8*). The yield of cultures can be substantially improved by inoculating clinical specimens into aerobic blood culture vials (BCVs) from a variety of commercial systems (*8*). Attempts to isolate the organism from synovial fluid or bone exudates on routine solid media succeeded in 2 of 25 patients, whereas inoculation of these specimens into aerobic BACTEC (Becton Dickinson, Cockeysville, MD, USA) BCVs yielded the organism in all cases after a median incubation of 4 days (*8*). When specimens from BCVs determined to be positive by the automated blood culture instrument were subcultured onto a blood–agar plate of trypticase soy agar with 5% sheep blood hemoglobin or chocolate agar, *K. kingae* grew readily, indicating that routine solid media are able to support the nutritional requirements of the organism. This observation suggests that skeletal system exudates exert a detrimental effect on this fastidious bacterium, and dilution of purulent material in a large volume of broth decreases the concentration of inhibitory factors, improving its recovery (*8*). In studies conducted in Israel and France in which BCVs were routinely inoculated with synovial fluid aspirates from young children who had arthritis, *K. kingae* was isolated in 48% of the patients with culture-proven disease (*8*,*9*). Conversely, when BCVs are not used, many *K. kingae* infections will be overlooked and labeled as culture-negative septic arthritis (*34*).

In recent years, development of NAAAs has further improved the diagnosis of *K. kingae* from skeletal system exudates. Use of this novel technique facilitates detection of difficult-to-culture organisms, enables bacteriologic diagnosis in patients already treated with antibacterial drugs, reduces time to detection, and facilitates precise identification of unusual species (*10*–*14*). The procedure consists of extraction of bacterial DNA from the synovial fluid sample, a PCR amplification step in which primers target the *16S rDNA*, the *23S rDNA*, or the *rpo* genes that are ubiquitous to all bacteria, then sequencing of the amplicon, and comparison of results with those kept in a broad database curator (such as GenBank) to enable precise species identification. Alternately, the specimen may be subjected to amplification by PCR by using species-specific primers that recognize the most plausible pathogens. Use of NAAAs has confirmed that in countries where this topic has been studied, *K. kingae* is the most common etiologic agent of septic arthritis in children <3 years of age. NAAA use

detected the presence of *K. kingae*–specific DNA sequences, including in cases in which seeding of synovial fluid specimens into BCVs failed to recover the organism and shortened the time required to detect and identify the bacterium from 3–4 days to <24 hours (*10*–*14*). It was, then, natural that this sensitive approach was consequently adopted to study respiratory colonization by *K. kingae* and its connection to invasive disease.

In an outbreak investigated by Bidet et al., the cause of the skeletal infections was determined by sequential use of real-time PCR targeting the *K. kingae*–specific toxin *rtx*A and the *cpn*60 genes (*35*). The same method was used to investigate the prevalence of *K. kingae* among attendees of the index daycare facility. The organism was recovered by culture in 6 asymptomatic carriers, whereas the NAAs detected 5 additional carriers (*35*). MLST and sequencing of the *rtx*A amplicon were performed directly on the joint fluid sample from the child who had arthritis and on the recovered pharyngeal isolates. All carriage isolates and the arthritis strain belonged to MLST 25 and shared *rtx*A allele 1, which is among the most common genotypes involved in joint and bone infections in France (*26*,*35*). It should be pointed out that, despite the increased sensitivity of NAAAs, when evaluating the efficacy of prophylactic antibacterial drug administration for eradicating *K. kingae* from colonized children, cultures have the obvious advantage of detecting living bacteria, whereas the viability of *K. kingae* organisms in PCR-positive/culture-negative specimens is questionable.

In addition detecting invasive *K. kingae* disease in patients, searching for asymptomatic but colonized children is crucial in the investigation of an outbreak in a daycare facility to assess the full extent of the contagion, identify attendees at risk for clinical disease, and evaluate the effects of prophylactic antibacterial drugs. Because of the high density of the resident bacterial flora and the relatively slow growth of *K. kingae*, detecting the organism in pharyngeal cultures is difficult. A differential and selective medium consisting of blood agar with 2 mg/mL of vancomycin (BAV medium) added has been developed to improve recovery of *K. kingae* from respiratory cultures (*36*). This formulation facilitates recognition of β-hemolytic *K. kingae* colonies by inhibiting growth of competitive gram-positive bacteria. In a blinded evaluation, the BAV medium detected 43 (97.7%) of 44 pharyngeal cultures positive for the organism; 10 (22.7%) positive cultures were identified on routine blood-agar plates ($p<0.001$) (*36*). If the original BAV formulation (*36*) or a similar medium (*23*) had not been used, and a chocolate-based agar substituted (in which the faint ring of hemolysis surrounding *K. kingae* colonies could not be recognized), carriers of the organism might not have been detected among 27 asymptomatic attendees of a daycare facility where a cluster of invasive disease occurred (*37*).

Daycare Centers as Reservoirs of Invasive *K. kingae* Disease

The *K. kingae* colonization rate is substantially enhanced among children in daycare centers. In an 11–month longitudinal study, 35 (72.9%) of 48 daycare center attendees carried the organism at least once and an average of 27.5% of the children were colonized at any given time (*20*). Molecular typing of isolates from asymptomatic colonized attendees showed genotypic similarities, indicating person-to-person transmission of the organism in the facility (*21*). Two *K. kingae* strains represented 28.0% and 46.0% of all isolates, demonstrating that some strains are particularly successful in colonizing mucosa. Children harbored the same strain continuously or intermittently for weeks or months, and then it was replaced by a new strain, showing that carriage is a dynamic process in which there is frequent turnover of colonizing organisms, as observed for other respiratory pathogens (*21*,*28*). Despite the high prevalence of the organism in the daycare center, an invasive *K. kingae* infection did not develop in any of the attendees in the course of the follow-up period.

The link between out-of-home child care and *K. kingae* carriage was recently confirmed in a study conducted among 1,277 children <5 years of age who were referred to a pediatric emergency department (*7*). Daycare attendance was strongly associated with *K. kingae* carriage after controlling for other variables (odds ratio 9.66 [95% CI 2.99–31.15], $p<0.001$) (*7*). Surveillance studies not only have showed that *K. kingae* organisms colonizing attendees of a given daycare center are frequently identical, but have also demonstrated that carried strains differ between facilities located close together, indicating that each daycare center is like an independent epidemiologic unit (*35*,*37*–*39*).

Considering these findings, it is not surprising that clusters of proven and presumptive cases of invasive *K. kingae* disease have been detected in daycare centers in France (*35*), the United States (*37*,*38*), and Israel (*39*), including 2 recent and still unreported outbreaks (P.Yagupsky, unpub. data) (Table 1). A presumptive case was defined as bacteriologically unconfirmed invasive disease, consistent with the clinical features of *K. kingae* infections, among daycare center attendees 6 months–3 years of age, within 1 month of an infection confirmed by culture and/or NAAAs. These events have been characterized by the simultaneous or consecutive occurrence of multiple cases of disease that included the entire clinical spectrum of *K. kingae* infections (septic arthritis, osteomyelitis, bacteremia, spondylodiscitis, cellulitis, and fatal meningitis complicating endocarditis). The 3 patients in the 2005 cluster in Israel (*39*) and 4 of the 5 patients reported in France (*35*) had bone infections, supporting the concept that certain *K. kingae* clones exhibit specific tissue tropism (*25*).

Table 1. Demographic and clinical features of 6 clusters of invasive *Kingella kingae* infections in daycare centers in the United States, Israel, and France*

Reference	Year	Country	Attack rate (%)	No. cases confirmed by		No. presump. cases	Patient age range, mo	Outbreak duration, d	Clinical syndromes						
				Culture	NAAA				SA	OM	SD	CE	OB	EN	MN
(*38*)	2003	United States	3/21 (14.3)	2	ND	1	17–21	<14	2†	2†	–	–	–	–	–
(*39*)	2005	Israel	3/14 (21.4)	1	ND	2	8–12	15	–	3	–	–	–	–	–
(*37*)	2007	United States	3/14 (21.4)	2	ND	1	11–25	11	–	–	1	1	–	1‡	1‡
(*35*)	2011	France	5/24 (20.8)	0	1	4	10–16	30	1	4	–	–	–	–	–
P. Yagupsky, unpub. data	2012	Israel	2/36 (5.6)	1	ND	1	10–16	7	1	–	–	–	1	–	–
P. Yagupsky, unpub. data	2013	Israel	2/13 (15.4)	2	ND	0	12	7	2	–	–	–	–	–	–
Total			18/122 (14.8)	8	1	9	8–25	7–30	6	9	1	1	1	1	1

*NAAA, nucleic acid amplification assay; presump., presumptive; SA, septic arthritis; OM, osteomyelitis; SD, spondylodiscitis; CE, cellulitis; OB, occult bacteremia; EN, endocarditis; MN, meningitis.; ND, not done; – indicates that no patients manifested this syndrome.
†One child had osteomyelitis of the femur and septic arthritis of the contiguous hip joint.
‡Meningitis developed in 1 child who had endocarditis.

Three of the 6 *K. kingae* illness outbreaks in daycare centers reported during 2003–2013 were detected in Israel, a small country with a population of 8,000,000 inhabitants(*35,37–39*; P.Yagupsky, unpub. data). Although Israel's relatively high annual birthrate compared with Western countries (18.7 per 1,000 population, Israel Central Bureau of Statistics, vs. 10.9 per 1,000 in the European Union, Demography Report 2010, Eurostat), and the widespread and early daycare center attendance could partially account for this observation, it seems plausible that similar events occur worldwide but are frequently overlooked.

The epidemiologic investigation of these outbreaks revealed that the *K. kingae* colonization rate among asymptomatic attendees to the daycare centers where clinical cases were detected was unusually high (up to 52.9% in a United States cluster, as determined by culture [*38*], and 68.8% in France, as demonstrated by a sensitive NAAA [*35*]) (Table 2), and all pharyngeal isolates detected in the classrooms where disease occurred were genotypically identical and indistinguishable from the patients' clinical isolates. The age of the colonized or infected daycare center attendees coincided with the age of increased susceptibility to *K. kingae* carriage and disease (*7*,*15*,*16*,*28*); the organism was detected in the pharyngeal culture of only1 of the caregivers, supporting the hypothesis that healthy adultsrarely carry *K. kingae* (*29*).

Despite a background carriage rate as high as 5%–12%, the incidence of invasive *K. kingae* infections reported in Israel was 9.4 per 100,000 children <5 years of age per year only (*16*), and the calculated annual risk of developing *K. kingae* osteomyelitis or septic arthritis for young carriers in Switzerland was <1% (*40*). When data from the 6 clusters of invasive disease detected in daycare centers are pooled, a documented or presumptive *K. kingae* infection developed in 1 in 7 classmates within a 1-month period (*35*,*37–39*), indicating that the outbreak strains combined enhanced colonization fitness, high transmissibility, and remarkable virulence.

The strain responsible for the cluster of osteomyelitis detected in Israel in 2005 (*39*) belonged to PFGE clone K and MLST-6 that ranked second among strains carried in

Table 2. Antibacterial drug prophylaxis administered and its effect on colonization rates and secondary invasive disease after *Kingella kingae* infections in daycare centers, United States, Israel, and France*

Reference	Initial carriage rate among healthy attendees (%)	Antibacterial drug prophylaxis				Interval between cultures, d	Post-prophylaxis carriage rate (%)	Post-prophylaxis new cases
		Rifampin		Amoxicillin				
		Dosage, 10 mg/kg 2×/d	Duration, d	Dosage, 40 mg/kg 2×/d	Duration, d			
(*38*)	9/17 (52.9)	Yes	2	No	NA	10–14	4/17 (23.5)	0/17
(*39*)	4/11 (36.4)	Yes	2	Yes	4	2	0/10	0/11
(*37*)	0/27	Yes	2	Yes	2	NA	ND	0/27
(*35*)	11/16 (68.8)†	Yes	2	No	NA	15	11/16† (68.8)	0/19
P. Yagupsky, unpub. data	4/36 (11.1)	Yes	2	Yes	4	10	2/36 (5.6)	0/36
P. Yagupsky, unpub. data	5/11 (45.4)	Yes	2	Yes	4	12	0/11	0/11
Total	33/118 (28.0)			NA			17/90 (18.9)	0/121

*ND, not done; NA, not applicable.
†As determined by a nucleic acid amplification assay. In all other cases carriage was established by culture on selective media.

southern Israel by healthy Jewish children (*20*), and was responsible for the excess of *K. kingae* illness observed in the Jewish population of the region over the previous 2 decades, causing 41.7% of all invasive strains isolated in this ethnic group (*27*). The clone that caused an outbreak in daycare centers in North Carolina, USA (*37*), represented 11.3% of all organisms carried by healthy children in southern Israel (*24*) and 11.6% of all isolates from patients in Israel who had invasive infections (*27*). The international distribution of these clones indicates that the clusters of disease are frequently caused by highly successful strains that exhibit enhanced capability for local and long-range dissemination (*26*).

Management of *K. kingae* Outbreaks

To prevent further cases of disease and eradicate the invasive strain, prophylactic antibacterial drugs have been administered to children attending the facilities where clusters of *K. kingae* were detected. Rifampin was chosen because *K. kingae* is especially susceptible to that antibacterial drug (*35*). Rifampin is secreted in saliva and reaches high concentrations in the upper respiratory mucosa and has shown efficacy in the eradication of colonization by *N. meningitidis* and *H. influenzae* type b, and in disease prevention in daycare centers (*2*). However, because only partial success was achieved with this drug in the Minnesota cluster (*38*), high-dose amoxicillin was added to the regimen after 2 more recent outbreaks (*37*,*39*). Following administration of antibacterial drugs, a respiratory carriage of the organism decreased, but complete eradication occurred only in the Durham, North Carolina daycare center (*37*), and new colonization of several attendees by the original strain was later observed (*35*,*38*,*39*). Persistence of the organism in the facility was not caused by bacterial resistance to the administered antibacterial drugs, however (*35*,*37*,*39*). Similar observations have been made in outbreaks in daycare centers caused by *H. influenzae* type b and pneumococci (*2*). Poor compliance or failure to administer prophylactic antibacterial drugs to family contacts could have resulted in incomplete suppression of the reservoir and recurrent dissemination of the strain in the facility (*2*).

Because antibacterial drugs have been relatively ineffective in eradicating *K. kingae* carriage, the need for antibacterial drug prophylaxis in the setting of a cluster of invasive disease is being disputed (*40*). Notably, however, after administration of antibacterial drugs to the asymptomatic children, no further cases of disease were detected in the affected daycare centers (*35*,*37*–*39*), even when a few children continue to carry the invasive strain. Reducing the bacterial density among colonized children by antibacterial drug administration or an effective immune response induced by prolonged mucosal carriage may have been sufficient to prevent new cases of infection. Improved hygiene and institution of other infection control measures could also have limited further transmission of the organism. The Figure depicts an algorithm aimed to guide the investigation and management of clusters of invasive *K. kingae* in daycare facilities, including areas of controversy.

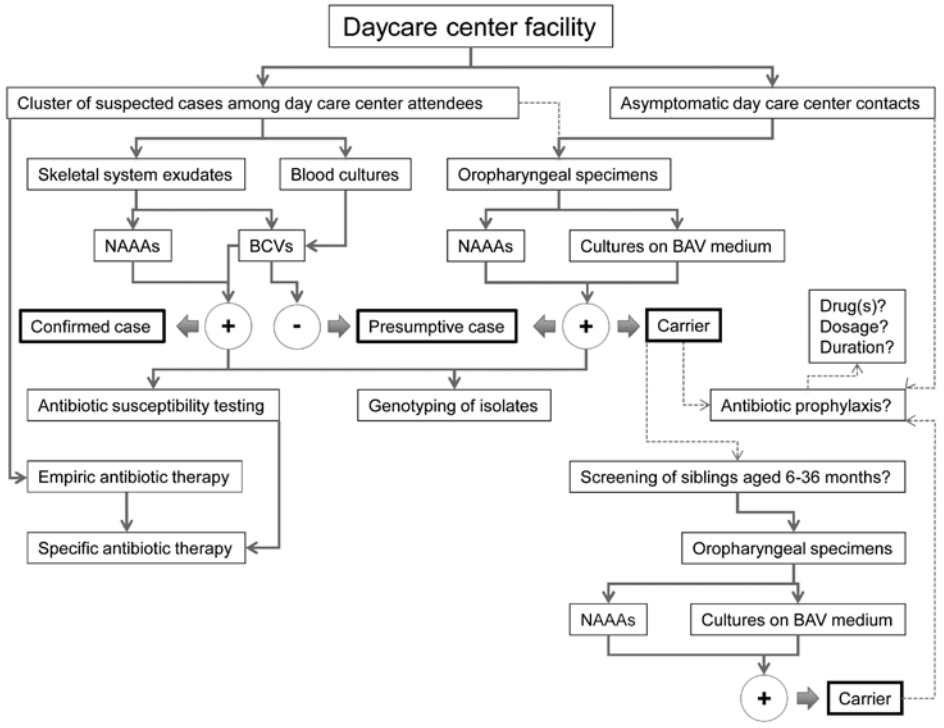


Figure. Suggested algorithm for the investigation and management of clusters of invasive *Kingella kingae* infections in daycare centers. Bold boxes indicate case type; dashed lines indicate controversial areas. NAAAs, nucleic acid amplification assays; BCVs, blood culture vials; BAV, blood-agar-vancomycin medium.

Conclusions

In recent years, clusters of invasive *K. kingae* infections among attendees of daycare centers have been reported, although because of the low rate of testing for this pathogen, many events are probably overlooked. Detection of these events requires a high level of clinical suspicion, use of sensitive culture techniques and NAAAs, and familiarity of the clinical microbiology laboratory with the identification of this elusive pathogen. The same improved detection methods should be employed for the thorough investigation of these clusters, and recovered *K. kingae* isolates should be genotyped and compared. Many issues remain unsettled, including whether antibacterial drugs should be administered prophylactically to daycare center contacts of an index case-patient, to young siblings of clinical case-patients, and to carriers, or whether antibacterial drugs should be offered to confirmed carriers only, or limited to children with disease. If administration of antibacterial drug prophylaxis is decided on, the preferred drug regimen will have to be determined. Whether an epidemiologic investigation should be carried out in daycare centers after detection of a single case of disease also remains to be determined.

Dr Yagupsky is a pediatrician and clinical microbiologist, and the head of the Clinical Microbiology Laboratory of the Soroka University Medical Center, Beer-Sheva, Israel. His main research interests include *Kingella kingae* infections, human brucellosis, and pediatric skeletal system infections.

References

1. Robinson J. Infectious diseases in schools and child care facilities. Pediatr Rev. 2001;22:39–46. http://dx.doi.org/10.1542/pir.22-2-39
2. Murphy TV, McCracken GH Jr, Moore BS, Gulig PA, Hansen HJ. *Haemophilus influenzae* type b disease after rifampin prophylaxis in a day care center: possible reasons for its failure. Pediatr Infect Dis. 1983;2:193–8. http://dx.doi.org/10.1097/00006454-198305000-00004
3. Weintraub A. Immunology of bacterial polysaccharide antigens. Carbohydr Res. 2003;338:2539–47. http://dx.doi.org/10.1016/j.carres.2003.07.008
4. Lu N, Samuels ME, Baker SL, Glover SH, Sanders JM. Child day care risks of common infectious diseases revisited. Child Care Health Dev. 2004;30:361–8. http://dx.doi.org/10.1111/j.1365-2214.2004.00411.x
5. Huskins WC. Transmission and control of infections in out-of-home child care. Pediatr Infect Dis J. 2000;19:S106–10. http://dx.doi.org/10.1097/00006454-200010001-00004
6. Osterholm MT. Infectious diseases in child day care: an overview. Pediatrics. 1994;94:987–90.
7. Amit U, Dagan R, Yagupsky P. Prevalence of pharyngeal carriage of *Kingella kingae* in young children and risk factors for colonization. Pediatr Infect Dis J. 2013;32:191–3. http://dx.doi.org/10.1097/INF.0b013e3182755779
8. Yagupsky P, Dagan R, Howard CW, Einhorn M, Kassis I, Simu A. High prevalence of *Kingella kingae* in joint fluid from children with septic arthritis revealed by the BACTEC blood culture system. J Clin Microbiol. 1992;30:1278–81.
9. Moumile K, Merckx J, Glorion C, Pouliquen JC, Berche P, Ferroni A. Bacterial aetiology of acute osteoarticular infections in children. Acta Paediatr. 2005;94:419–22. http://dx.doi.org/10.1080/08035250410023278
10. Cherkaoui A, Ceroni D, Emonet S, Lefevre Y, Schrenzel J. Molecular diagnosis of *Kingella kingae* osteoarticular infections by specific real-time PCR assay. J Med Microbiol. 2009;58:65–8. http://dx.doi.org/10.1099/jmm.0.47707-0
11. Moumile K, Merckx J, Glorion C, Berche P, Ferroni A. Osteoarticular infections caused by *Kingella kingae* in children: contribution of polymerase chain reaction to the microbiologic diagnosis. Pediatr Infect Dis J. 2003;22:837–9. http://dx.doi.org/10.1097/01.inf.0000083848.93457.e7
12. Lehours P, Freydière AM, Richer O, Burucoa C, Boisset S, Lanotte F, et al. The *rtx*A toxin gene of *Kingella kingae*: a pertinent target for molecular diagnosis of osteoarticular infections. J Clin Microbiol. 2011;49:1245–50. http://dx.doi.org/10.1128/JCM.01657-10
13. Chometon S, Benito Y, Chaker M, Boisset S, Ploton C, Bérard J, et al. Specific real-time polymerase chain reaction places *Kingella kingae* as the most common cause of osteoarticular infections in young children. Pediatr Infect Dis J. 2007;26:377–81. http://dx.doi.org/10.1097/01.inf.0000259954.88139.f4
14. Ilharreborde B, Bidet P, Lorrot M, Even J, Mariani-Kurkdjian P, Ligouri S, et al. New real-time PCR-based method for *Kingella kingae* DNA detection: application to samples collected from 89 children with acute arthritis. J Clin Microbiol. 2009;47:1837–41. http://dx.doi.org/10.1128/JCM.00144-09
15. Yagupsky P, Bar-Ziv Y, Howard CB, Dagan R. Epidemiology, etiology, and clinical features of septic arthritis in children younger than 24 months. Arch Pediatr Adolesc Med. 1995;149:537–40. http://dx.doi.org/10.1001/archpedi.1995.02170180067010
16. Dubnov-Raz G, Ephros M, Garty BZ, Schlesinger Y, Maayan-Metzger A, Hasson J, et al. Invasive pediatric *Kingella kingae* infections: a nationwide collaborative study. Pediatr Infect Dis J. 2010;29:639–43. http://dx.doi.org/10.1097/INF.0b013e3181d57a6c
17. Porsch EA, Kehl-Fie TE, Geme JW 3rd. Modulation of *Kingella kingae* adherence to human epithelial cells by type IV pili, capsule, and a novel trimeric autotransporter. mBio 2012; 3:e00372–12. PubMed http://dx.doi.org/10.1128/mBio.00372-12
18. Starr KF, Porsch EA, Heiss C, Black I, Azadi P, St Geme JW III. Characterization of the *Kingella kingae* polysaccharide capsule and exopolysaccharide. PLoS ONE. 2013;8:e75409. http://dx.doi.org/10.1371/journal.pone.0075409
19. Kehl-Fie TE, St Geme JW III. Identification and characterization of an RTX toxin in the emerging pathogen *Kingella kingae.* J Bacteriol. 2007;189:430–6. http://dx.doi.org/10.1128/JB.01319-06
20. Yagupsky P, Dagan R, Prajgrod F, Merires M. Respiratory carriage of *Kingella kingae* among healthy children. Pediatr Infect Dis J. 1995;14:673–77. http://dx.doi.org/10.1097/00006454-199508000-00005
21. Slonim A, Walker ES, Mishori E, Porat N, Dagan R, Yagupsky P. Person-to-person transmission of *Kingella kingae* among day care center attendees. J Infect Dis. 1998;178:1843–46. http://dx.doi.org/10.1086/314488
22. Yagupsky P, Porat N, Pinco E. Pharyngeal colonization by *Kingella kingae* in children with invasive disease. Pediatr Infect Dis J. 2009;28:155–7. http://dx.doi.org/10.1097/INF.0b013e318184dbb8
23. Basmaci R, Ilharreborde B, Bidet P, Doit C, Lorrot M, Mazda K, et al. Isolation of *Kingella kingae* in the oropharynx during *K. kingae* arthritis on children. Clin Microbiol Infect. 2012;18:e134–6. http://dx.doi.org/10.1111/j.1469-0691.2012.03799.x
24. Yagupsky P, Weiss-Salz I, Fluss R, Freedman L, Peled N, Trefler R, et al. Dissemination of *Kingella kingae* in the community and long-term persistence of invasive clones. Pediatr Infect Dis J. 2009;28:707–10. http://dx.doi.org/10.1097/INF.0b013e31819f1f36

25. Amit U, Porat N, Basmaci R, Bidet P, Bonacorsi S, Dagan R, et al. Genotyping of invasive *Kingella kingae* isolates reveals predominant clones and association with specific clinical syndromes. Clin Infect Dis. 2012;55:1074–9. http://dx.doi.org/10.1093/cid/cis622
26. Basmaci R, Yagupsky P, Ilharreborde B, Guyot K, Porat N, Chomton M, et al. Multilocus sequence typing and *rtxA* toxin gene sequencing analysis of *Kingella kingae* isolates demonstrates genetic diversity and international clones. PLoS ONE. 2012;7:e38078. http://dx.doi.org/10.1371/journal.pone.0038078
27. Amit U, Dagan R, Porat N, Trefler R, Yagupsky P. Epidemiology of invasive *Kingella kingae* infections in two distinct pediatric populations cohabiting in one geographic area. Pediatr Infect Dis J. 2012;31:415–7. http://dx.doi.org/10.1097/INF.0b013e318240cf8a
28. Amit U, Flaishmakher S, Dagan R, Porat, N, Yagupsky P. Age-dependent carriage of *Kingella kingae* in young children and turnover of colonizing strains. J Pediatr Infect Dis Soc. In press. [cited 2014 Mar 5]. http://dx.doi.org/10.1093/jpids/pit003
29. Yagupsky P, Peled N, Katz O. Epidemiological features of invasive *Kingella kingae* infections and respiratory carriage of the organism. J Clin Microbiol. 2002;40:4180–4. http://dx.doi.org/10.1128/JCM.40.11.4180-4184.2002
30. Slonim A, Steiner M, Yagupsky P. Immune response to invasive *Kingella kingae* infections, age-related incidence of disease, and levels of antibody to outer-membrane proteins. Clin Infect Dis. 2003;37:521–7. http://dx.doi.org/10.1086/376913
31. Basmaci R, Lorrot M, Bidet P, Doit C, Vitoux C, Penneçot G, et al. Comparison of clinical and biologic features of *Kingella kingae* and *Staphylococcus aureus* arthritis at initial evaluation. Pediatr Infect Dis J. 2011;30:902–4. http://dx.doi.org/10.1097/INF.0b013e31821fe0f7
32. Ceroni D, Cherkaoui A, Combescure C, Francois P, Kaelin A, Schrenzel J. Differentiating osteoarticular infections caused by *Kingella kingae* from those due to typical pathogens in young children. Pediatr Infect Dis J. 2011;30:906–9. http://dx.doi.org/10.1097/INF.0b013e31821c3aee
33. Baraff LJ, Bass JW, Fleisher GR, Klein JO, McCracken GH, Powell KR, et al. Practice guideline for the management of infants and children 0 to 36 months of age with fever without source. Agency for Health Policy and Research. Ann Emerg Med. 1993;22:1198–210. http://dx.doi.org/10.1016/S0196-0644(05)80991-6
34. Infectious Diseases Society of America Emerging Infections Network. Brief Report: *Kingella kingae* infections in children—United States, June 2001–November 2002. MMWR Morb Mortal Wkly Rep. 2004;53:244.
35. Bidet P, Collin E, Basmaci R, Courroux C, Prisse V, Dufour V, et al. Investigation of an outbreak of osteoarticular infections caused by *Kingella kingae* in a childcare center using molecular techniques. Pediatr Infect Dis J. 2013;32:558–60. http://dx.doi.org/10.1097/INF.0b013e3182867f5e
36. Yagupsky P, Merires M, Bahar J, Dagan R. Evaluation of a novel vancomycin-containing medium for primary isolation of *Kingella kingae* from upper respiratory tract specimens. J Clin Microbiol. 1995;33:1426–7.
37. Seña AC, Seed P, Nicholson B, Joyce M, Cunningham CK. *Kingella kingae* endocarditis and a cluster investigation among daycare attendees. Pediatr Infect Dis J. 2010;29:86–8. http://dx.doi.org/10.1097/INF.0b013e3181b48cc3
38. Kiang KM, Ogunmodede F, Juni BA, Boxrud DJ, Glennen A, Bartkus JM, et al. Outbreak of osteomyelitis/septic arthritis caused by *Kingella kingae* among child care center attendees. Pediatrics. 2005;116:e206–13. http://dx.doi.org/10.1542/peds.2004-2051
39. Yagupsky P, Erlich Y, Ariela S, Trefler R, Porat N. Outbreak of *Kingella kingae* skeletal system infections in children in daycare. Pediatr Infect Dis J. 2006;25:526–32. http://dx.doi.org/10.1097/01.inf.0000215243.42501.4f
40. Ceroni D, Dubois-Ferrière V, Anderson R, Combescure C, Lamah, Cherkaoui A, et al. Small risk of osteoarticular infections in children with asymptomatic carriage of *Kingella kingae.* Pediatr Infect Dis J. 2012;31:983–5. http://dx.doi.org/10.1097/INF.0b013e31825d3419

Address for correspondence: Pablo Yagupsky, Clinical Microbiology Laboratory, Soroka University Medical Center, Ben-Gurion University of the Negev, Beer-Sheva 84101, Israel; email: yagupsky@bgu.ac.il

Molecular Investigation of Tularemia Outbreaks, Spain, 1997–2008

Jaime Ariza-Miguel, Anders Johansson, María Isabel Fernández-Natal, Carmen Martínez-Nistal, Antonio Orduña, Elías F. Rodríguez-Ferri, Marta Hernández, and David Rodríguez-Lázaro

Tularemia outbreaks occurred in northwestern Spain in 1997–1998 and 2007–2008 and affected >1,000 persons. We assessed isolates involved in these outbreaks by using pulsed-field gel electrophoresis with 2 restriction enzymes and multilocus variable number tandem repeat analysis of 16 genomic loci of *Francisella tularensis,* the cause of this disease. Isolates were divided into 3 pulsotypes by pulsed-field gel electrophoresis and 8 allelic profiles by multilocus variable number tandem repeat analysis. Isolates obtained from the second tularemia outbreak had the same genotypes as isolates obtained from the first outbreak. Both outbreaks were caused by genotypes of genetic subclade B.Br:FTNF002–00, which is widely distributed in countries in central and western Europe. Thus, reemergence of tularemia in Spain was not caused by the reintroduction of exotic strains, but probably by persistence of local reservoirs of infection.

Tularemia is a zoonosis caused by the gram-negative bacterium *Francisella tularensis*. *F. tularensis* is a highly contagious facultative intracellular pathogen and has infectious doses as low as 10–50 bacteria; it is transmitted by inhalation, direct contact with infected animals, or ingestion of contaminated water or food. The number of species susceptible to infection by this agent is higher than for any other known zoonotic pathogen (*1*). Because of its potential to cause adverse public health effects and mass casualties by bioterrorist attack, the pathogen is 1 of 6 agents listed as a Tier 1 agent by the US Department of Health and Human Services (*2*).

Author affiliations: Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain (J. Ariza-Miguel, M. Hernández, D. Rodríguez-Lázaro); Umeå University, Umeå, Sweden (A. Johansson); Complejo Asistencial Universitario de León, León, Spain (M.I. Fernández-Natal); Laboratorio Regional de Sanidad Animal León, Valladolid (C. Martínez-Nistal); Universidad de Valladolid, Valladolid (A. Orduña); Universidad de León, León (E.F. Rodríguez-Ferri); and Universidad de Burgos, Burgos, Spain (D. Rodríguez-Lázaro)

DOI: http://dx.doi.org/10.3201/eid2005.130654

F. tularensis includes 4 subspecies (*F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *novicida*, and *F. tularensis* subsp. *mediasiatica*), which show marked differences in many epidemiologic features, including geographic distribution, virulence, and genetic diversity (*3*). *F. tularensis* subsp. *tularensis* (Jellison type A) and *F. tularensis* subsp. *holarctica* (Jellison type B) are major clinical pathogens. *F. tularensis* subsp. *tularensis* is the most virulent subspecies and can cause life-threatening disease; its distribution seems to be restricted to North America, although a single report indicated its presence in Europe (*4–7*). *F. tularensis* subsp. *holarctica* causes a less severe disease, and although widespread throughout the Northern Hemisphere, it has restricted genetic diversity, which suggests recent emergence and successful geographic spread (*5,7–9*).

Tularemia was first reported in Spain in 1997, when it caused one of the largest outbreaks in humans ever described (*10*). Overall, 559 cases were confirmed during June 1997–April 1998 in 10 provinces. The outbreak was associated with hunting and handling of hares (*Lepus europaeus*) in northwestern Spain. The most common clinical form was ulceroglandular tularemia (55.4%); glandular (15.3%) and typhoid forms (6.6%) of the disease also occurred frequently. A second major human outbreak in humans, which affected 507 persons, occurred in the same area in 2007 and 2008, but in a different epidemiologic context. Its timing coincided with a population peak of the common vole (*Microtus arvalis*), and the most frequent clinical forms of the disease were typhoidal and pneumonic (65% of the cases), which is consistent with infection being acquired through inhalation of *F. tularensis* (*11–13*). Sporadic tularemia cases and small outbreaks were reported during 2000–2006 in the interval between the 2 major outbreaks in northwestern Spain (*13,14*).

We report comparative genetic analyses of *F. tularensis* cultured from humans and animals during the 2 main tularemia outbreaks (1997–1998 and 2007–2008). We also

studied *F. tularensis* isolates circulating in Spain during outbreaks with different epidemiologic patterns and investigated whether reemergence of the pathogen after 10 years of no epidemiologic activity was caused by introduction of exotic strains or by establishment of the pathogen in local reservoirs of infection.

Methods

F. tularensis Isolates, Culture Conditions, and Biochemical Characterization

We studied 109 *F. tularensis* isolates: 37 animal and human *F. tularensis* subsp. *holarctica* isolates from the first outbreak in northwestern Spain (1997–1998); 61 animal and human isolates from the second tularemia epidemic in the same area (2007–2008); 10 10 isolates obtained in the Czech Republic; and reference strain *F. tularensis* subsp. *tularensis* Schu (CAPM 5600). Source of isolates, subspecies, host, geographic origin, and year of isolation are shown in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/5/13-0654-Techapp1.xlsx).

All isolates were grown on modified Thayer-Martin agar plates containing 36 g/L GC agar base, 10 g/L soluble hemoglobin powder, and 2 vials/L Vitox supplement (Oxoid, Basingstoke, UK) at 37°C for 2–3 days in aerobic conditions. Biochemical characterization included tests for oxidase and catalase activities, glucose and glycerol fermentation, and urea hydrolysis.

Genetic Characterization

Species and subspecies were identified by real-time and conventional PCRs specific for the *fopA* gene and the region of difference 1 (RD1) as described (*15,16*). RD23 was analyzed by PCR for identification of a genetic group of isolates that had been found on the Iberian Peninsula (*17*).

Pulsed-field gel electrophoresis (PFGE) and multilocus variable number tandem repeat analysis (MLVA) were used to classify isolates into genetic subpopulations. The PFGE protocol described (*18*), which used restriction enzymes *Xho*I and *Bam*HI, was optimized to provide major improvements in quality of fingerprint patterns.

Bacterial cells were suspended in SE buffer (25 mmol/L EDTA, 75 mmol/L NaCl, pH 7.5) to an absorbance of 0.5–0.6 at 600 nm. Cells were lysed in agarose plugs and plugs were washed 5 times with Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) for 30 min at 50°C. DNA in the plugs was digested with 40 U of *Xho*I (New England Biolabs, Ipswich, MA, USA) or 40 U of *Bam*HI (New England Biolabs), for 16 and 3 h, respectively, at 37°C following the manufacturer's protocol. DNA fragment sizes were determined by electrophoresis and by comparing bands with a Lambda Ladder PFG Marker (New England Biolabs).

MLVA was performed as described for 16 variable number tandem repeat loci (*5*). To ensure analysis of identical genetic material by PFGE and MLVA, we used DNA from the same culture for both methods. MLVA markers were amplified by using PCR, and sizes of amplification products were determined by electrophoresis on 3.5% high-resolution agarose MS-8 gels (Conda Pronadisa, Madrid, Spain), except for Ft-M3, Ft-M21, Ft-M22, and Ft-M24 MLVA markers, for which the sizes were determined by using capillary electrophoresis. At least 2 alleles were sequenced for each MLVA marker to confirm that size differences observed resulted from the expected variations in numbers of tandem repeats. Forward and reverse sequences were aligned by using MEGA v.4 software (*19*), and consensus sequences were used to predict the number of tandem repeats in each allele.

Data Analyses

The Simpson index of diversity, which measures the probability that 2 unrelated strains from the test population will be classified into different typing groups (*20*), was calculated to compare the discriminative power of PFGE typing with that of MLVA for assessing genetic diversity among isolates. The adjusted Wallace coefficient for quantification of agreement between PFGE typing and MLVA results was also calculated. Both analyses were performed by using Comparing Partitions (*21*).

PFGE patterns were analyzed by using Bionumerics v.6.6 (Applied-Maths NV, Sint-Martens-Latem, Belgium) to describe genetic relationships among isolates. Dendrograms were constructed by using the Dice similarity coefficient and the unweighted pair group mathematical average clustering algorithm. MLVA data, expressed as allelic profiles for isolates, were analyzed by using Bionumerics v.6.6. Minimum spanning trees were calculated with priority rules set at first link allelic profiles and maximum numbers of single-locus variants and then maximal numbers of single-locus variants and double-locus variants. MLVA types were classified as members of a clonal complex if they had the same allele at15 of the 16 MLVA markers. A map of the distribution of isolates showing the geographic origin and number of isolates per province was generated by using Arcgis v.9.2 software (ESRI, Redlands, CA, USA).

Results

Subspecies and Genetic Subclade of *F. tularensis* Isolates

All isolates were negative for oxidase activity, weakly positive for catalase activity, and positive for acid production from glucose; none of the isolates hydrolyzed urea. Only the reference strain *F. tularensis* subsp. *tularensis*

Schu (CAPM 5600) produced acid from glycerol. Real-time PCR specific for the *fop*A gene and size determination at the RD1 region showed that all isolates from Spain and the Czech Republic were *F. tularensis* subsp. *holarctica* (online Technical Appendix). All isolates from Spain included in this study had the 1.59-kbp deletion at the RD23 loci, which is characteristic of the *F. tularensis* subsp. *holarctica* genetic subclade B.Br:FTNF002–00 (also known as the Iberian clone or the central and western European genetic group).

Characterization by PFGE

All 107 *F. tularensis* subsp. *holarctica* isolates showed the same fingerprint pattern by PFGE with the restriction enzyme *Xho*I, irrespective of their geographic origin, host, or date of isolation (isolate TU41 was not typeable by PFGE analysis). This pattern consisted of ≈20 DNA fragments >70 kbp. The *F. tularensis* subsp. *tularensis* strain Schu (CAPM 5600) showed a different banding pattern.

In contrast, PFGE with the restriction enzyme *Bam*HI discriminated 5 genotypes among the *F. tularensis* subsp. *holarctica* isolates. *F. tularensis* subsp. *tularensis* strain Schu (CAPM 5600) showed a highly unrelated banding pattern with maximal pairwise distance to all other isolates. The *Bam*HI patterns consisted of 20–24 DNA fragments with a size range of 20–245 kbp. All *F. tularensis* subsp. *holarctica* genotypes were closely related (93.3% similarity), and there were only 1-band differences between pulsotypes (Figure 1, Appendix, wwwnc.cdc.gov/EID/article/20/5/13-0654-F1.htm).

Sixty-nine (63.9%) isolates from Spain clustered into pulsotype A and 26 (24.1%) other isolates from Spain clustered into pulsotype B. All isolates from the Czech Republic, except for isolate CAPM 5538, had the same fingerprint pattern, which was designated pulsotype D (8.3%). Isolate CAPM 5538 showed a pulsotype that clustered with the 2 remaining isolates from Spain (TU8 and TU9) in pulsotype C (1.9%). One isolate from Spain, TU41, could not be genotyped by PFGE despite several attempts (online Technical Appendix).

There were some discrepancies between our findings and those reported for the 37 isolates in Spain from 1997 (*18*). Improvements in quality of fingerprint patterns enabled us to distinguish between isolates from Spain and those from the Czech Republic by using PFGE and restriction enzyme *Bam*HI. Furthermore, isolates TU3, TU17, TU21, and TU25 were unequivocally assigned to pulsotype B instead of pulsotype A. In instances of discrepancy, analyses were repeated in triplicate with new cultures, and the findings reported were confirmed. Distribution of the 107 *F. tularensis* subsp. *holarctica* isolates into 4 pulsotypes resulted in a Simpson index of diversity of 0.522. There was no obvious correlation between the pulsotype of an isolate from Spain and its geographic origin, host, or tularemia outbreak with which it was associated.

Characterization by MLVA

The allele-based analysis of genetic relationships identified 13 MLVA types among the 108 *F. tularensis* subsp. *holarctica* isolates and showed that *F. tularensis* subsp. *tularensis* strain Schu (CAPM 5600) was more distantly related (Figure 2). The 10 isolates from the Czech Republic were assigned to 5 MLVA types, which differed from isolates from Spain by ≥2 alleles. Marker Ft-M3 provided the highest number of alleles (6). Six copy numbers were detected among the 109 isolates; for Ft-M6, Ft-M9, and Ft-M20, there were 3 copy numbers. Markers Ft-M5, Ft-M7, Ft-M8, Ft-M10, Ft-M13, FT-M16, Ft-M19, Ft-M21, Ft-M22, Ft-M23, and Ft-M24 each had 2 alleles: these markers with 2 alleles, except for Ft-M24, discriminated only *F. tularensis* subsp. *tularensis* strain Schu (CAPM 5600) from all isolates of *F. tularensis* subsp. *holarctica* (Table).

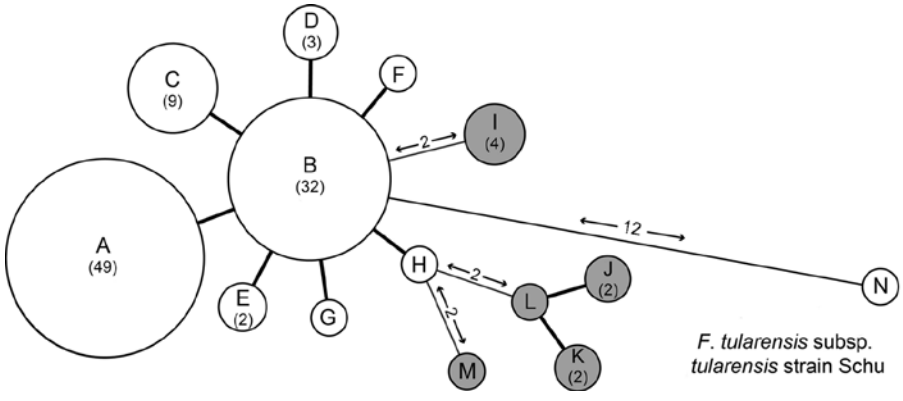


Figure 2. Minimum-spanning tree based on multilocus variable number of tandem repeat analysis (MLVA) genotypes, showing genetic relationships among 98 *Francisella tularensis* subsp. *holarctica* isolates from Spain (white circles), 10 *F. tularensis* subsp. *holarctica* reference isolates from the Czech Republic (gray circles), and reference strain *F. tularensis* subsp. *tularensis* Schu (CAPM 5600). Each node represents a unique MLVA type, and size is proportional to the number of isolates with that genotype (values in parentheses). Numbers on lines between nodes indicate number of typing markers that were different between genotypes. A 1-marker difference is indicated by a thick line.

Table. Multilocus variable number tandem repeat analysis of 98 *Francisella tularensis* isolates from Spain and 11 reference isolates*

MLVA genotype	No. isolates (origin)	No. MLVA markers that discriminated *F. tularensis* subsp. *holarctica* isolates				
		Ft-M3	Ft-M6	Ft-M9	Ft-M20	Ft-M24†
A	49 (Spain)	5	4	2	3	2 (Δ16 bp)
B	32 (Spain)	4	4	2	3	2 (Δ16 bp)
C	9 (Spain)	6	4	2	3	2 (Δ16 bp)
D	3 (Spain)	3	4	2	3	2 (Δ16 bp)
E	2 (Spain)	4	7	2	3	2 (Δ16 bp)
F	1 (Spain)	4	4	2	4	2 (Δ16 bp)
G	1 (Spain)	4	4	3	3	2 (Δ16 bp)
H	1 (Spain)	7	4	2	3	2 (Δ16 bp)
I	4 (former Czechoslovakia)	4	6	2	3	2
J	2 (Czech Republic)	5	7	2	3	2
K	2 (Czech Republic)	6	7	2	3	2
L	1 (former Czechoslovakia)	7	4	2	2	2
M	1 (Czech Republic)	7	7	2	3	2
N‡	1 (United States)	28	4	4	3	1

*MLVA, multilocus variable number tandem repeat.
†All isolates from Spain had the unique 16-bp deletion at marker FT-M24 that is characteristic of genetic subclade B.Br:FTNF002–00 (Iberian clone).
‡Genotype N, *F. tularensis* subsp. *tularensis* strain Schu, showed additional unique alleles at the following MLVA markers: Ft-M5, Ft-M7, Ft-M8, Ft-M10, Ft-M13, Ft-M16, Ft-M19, Ft-M21, Ft-M22, and Ft-M23.

Ft-M24 had a 464-bp allele that was found in all isolates from Spain analyzed in this study, but was not present in any other isolates. Ft-M24 has been found only in isolates of genetic subclade B.Br:FTNF002–00 (the Iberian clone or the central and western European genetic group). Sequence analysis of the Ft-M24 DNA fragment showed that the unique allele size was caused by deletion of a 16-bp sequence adjacent to 2 copies of the Ft-M24 tandem repeat (GenBank accession no. KC696513). For marker Ft-M12, because all 109 isolates had the same copy number, this marker provided no typing resolution. Distribution of *F. tularensis* subsp. *holarctica* isolates among 12 MLVA types was uneven; >70% of the isolates in the MLVA types A (49 isolates, 45%) and B (32 isolates, 29.4%) (Table). Simpson index of diversity, which showed the discriminatory power of MLVA for the 108 *F. tularensis* subsp. *holarctica* isolates, was 0.708.

The 98 isolates from Spain were classified into 8 MLVA types, which essentially grouped as 2 closely related clonal complexes that differed at only 1 of the 16 MLVA markers (Figure 2). All MLVA types from Spain were single-locus variants of MLVA type B, which indicated this type was the founder genotype of *F. tularensis* that caused tularemia in northwestern Spain. No clear relationship was found between genotype and geographic origin (Figure 3), source of infection, or host in Spain. The same genotype was usually isolated from hares, voles, and humans in Spain.

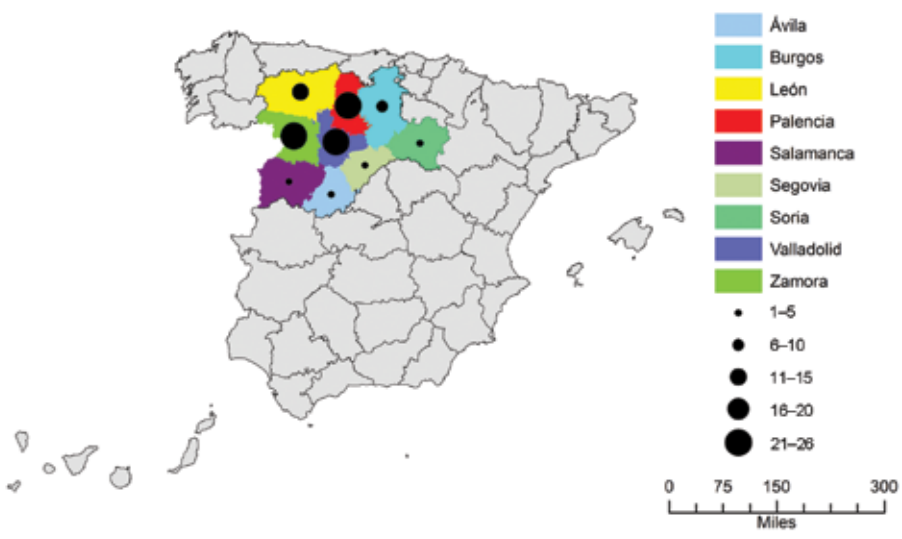
Comparison of isolates from the 2 outbreak periods (37 isolates for 1997–1998 and 61 isolates for 2007–2008) showed that the same *F. tularensis* genotypes caused tularemia in both outbreaks (Figure 4). Isolates from the second outbreak showed less genetic diversity than those from the first outbreak (Simpson indices 0.62, 95% CI 0.53–0.71 and 0.66, 95% CI 0.57–0.75, respectively; the difference was not significant at the 95% level). Comparison of allele distribution at the most variable marker (Ft-M3) showed an overall similarity between isolates causing the outbreaks, although the most common copy number was 4 during the first outbreak and 5 during the second outbreak, which might indicate a stepwise increase in copy number over time. Overall, our findings for 37 isolates from the first outbreak were consistent with the data reported by Dempsey et al. (*17*) although there were 2 discrepancies. First, isolate TU18 had a unique allele with 3 tandem repeats at Ft-M9, which distinguished this isolate from all other *F. tularensis* subsp. *holarctica* isolates. Second, isolate TU31 had the same FT-M10 allele as all other isolates. We confirmed our results for these discrepancies in triplicate.

Quantification of Agreement between PFGE Typing and MLVA

Congruence of the 2 methods (PFGE typing and MLVA) for isolate classification was weak for the 107 *F. tularensis* subsp. *holarctica* isolates (1 of the 108 isolates was excluded because it was not typeable by PFGE analysis). This finding was true for reverse comparisons of both methods: if 2 isolates were in the same PFGE pulsotype; they had an 18% chance of having the same MLVA type. Conversely, having the same MLVA type was associated with a 40% chance of having the same PFGE pulsotype. The adjusted Wallace coefficient for PFGE versus MLVA was 0.18 (95% CI 0.04–0.32) and that for MLVA versus PFGE was 0.40 (95% CI 0.20–0.58).

Discussion

F. tularensis subsp. *holarctica* has shown limited genetic diversity worldwide (*5,7,22,23*). This finding might be the result of a relatively recent bottleneck or clonal expansion event that drastically reduced genetic variation of the bacterial population (*5,7*). Consistent with previous



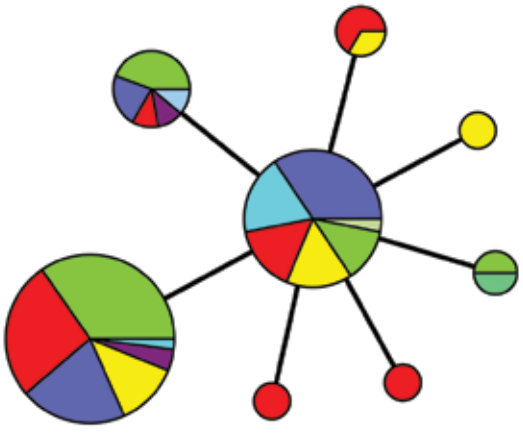


Figure 3. A) Geographic distribution of 98 *Francisella tularensis* subsp. *holarctica* isolates from Spain. Color codes represent geographic origin, and black circles represent number of isolates recovered per province. B) Minimum-spanning tree based on multilocus variable number tandem repeat (MLVA) analysis of genotypes, showing genetic relationships among 98 *F. tularensis* subsp. *holarctica* isolates from Spain. Each circle represents a unique MLVA type and size of each node is proportional to the number of isolates of that type. The MLVA types are colored according to the geographic origins of the isolates with the same color code as in panel A.

findings, we observed extremely limited genomic diversity among the 98 *F. tularensis* subsp. *holarctica* isolates from Spain analyzed by 2 genotyping tools: PFGE with 2 restriction enzymes and MLVA at 16 highly variable tandem repeat loci. PFGE identified 3 genotypes with single band differences (93.3% similarity) (Figure 1). MLVA discriminated these isolates into 8 MLVA types, but in pairwise comparisons they differed at no more than 2 of 16 MLVA markers. Thus, our collection shows extreme genetic homogeneity (Figure 2).

An *F. tularensis* subsp. *holarctica* lineage in central and western Europe (France, Spain, and Switzerland) has been defined (*7,17,24,25*). Strains belonging to this lineage have 2 unique genetic traits: a 1.59-kbp genomic deletion at the RD23 locus and a unique 464-bp allele at Ft-M24. All isolates from Spain had these alleles, irrespective of the outbreak, geographic origin, or the host from which they were recovered. Thus, all isolates from Spain analyzed belong to the genetic subclade B.Br:FTNF002–00 (the Iberian clone or central and western European genetic group) (*7*). Furthermore, all MLVA types for isolates from Spain were single-locus variants of MLVA type B, which suggested that this type might be a founding genotype that has evolved into multiple other genotypes that differ from the founding genotype at a single loci. In this scenario, all strains causing tularemia outbreaks in Spain are linked to this founder (ancestral) genotype.

We found poor congruence between typing results of PFGE and MLVA for 107 *F. tularensis* subsp. *holarctica* isolates. However, the 2 methods might indicate different types of genetic variation. PFGE is a suitable approach for detecting rearrangements in a genome, and differences of only 1 band observed among the 98 isolates from Spain are presumably consequences of a single mutation event that might be an inversion, translocation, deletion, or a single-nucleotide polymorphism (*26*). In contrast, MLVA detects variation in several, fast-evolving, repeated sequences. However, such rapidly evolving sequences are susceptible to homoplasy, and genetic classification of isolates on the basis of a difference at only 1 of 16 genetic loci, as for isolates from Spain, could be biased because of genetic reversion events.

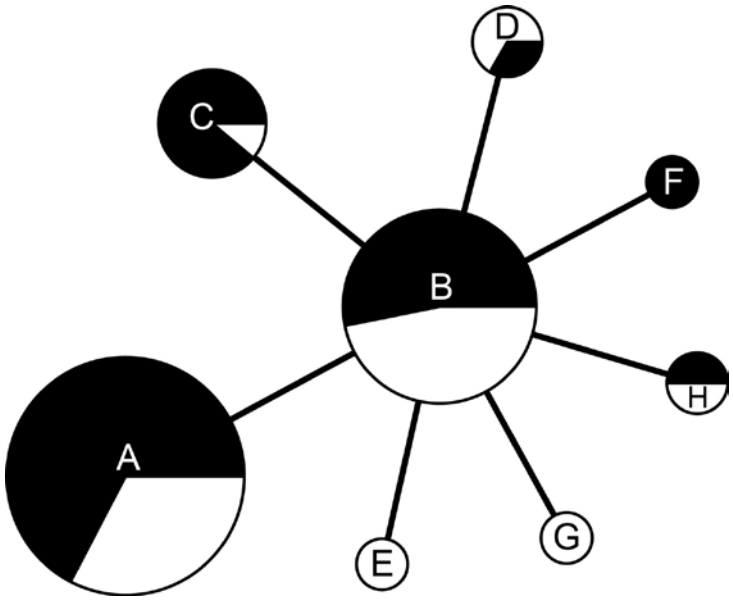


Figure 4. Minimum-spanning tree based on multilocus variable number tandem repeat (MLVA) analysis of genotypes showing genetic relationships among 98 *Francisella tularensis* subsp. *holarctica* isolates from Spain with reference to 2 human tularemia outbreaks in 1997–1998 and 2007–2008, respectively. White sections in circles indicate *F. tularensis* subsp. *holarctica* isolates recovered during the first human tularemia outbreak (1997–1998), and black sections indicate isolates recovered during the second outbreak (2007–2008). Each circle represents a unique MLVA type and size is proportional to the number of isolates of that type.

Because some of the mutations detected by PFGE or MLVA might not be selectively neutral, we might have observed time-dependent mutations that are transient on evolutionary time scales and will frequently be eliminated by selection pressure acting on them (*27*). If this hypothesis is true, use of single mutations for genetic discrimination may lead to incorrect phylogenetic inferences. Therefore, poor congruence between PFGE and MLVA for identifying genetic subclade B.Br:FTNF002–00 of *F. tularensis* subsp. *holarctica* in Spain might be caused by limited bacterial diversity. Use of more extensive genetic analyses for typing, such as whole genome sequencing, might be useful in subsequent molecular epidemiology studies.

In Spain, tularemia was first reported in late 1997 in association with one of the largest human outbreaks ever described (*10*). The most common route of infection of humans was by direct contact when hunting and handling hares (*L. europaeus*). Consistent with infection with *F. tularensis* through the skin, the most frequent clinical form was ulceroglandular tularemia (55.4%); glandular (15.3%) and typhoid (6.6%) forms of the disease were also observed. A second major human outbreak occurred in the same geographic area in northwest Spain in 2007 and 2008 after 10 years of no epidemiologic activity. The epidemiology of the second outbreak was different from that of the first outbreak. The second outbreak occurred when the population of the common vole (*Microtus arvalis*) peaked, and >65% of case-patients had typhoidal and pneumonic forms of tularemia (*12,13*), which is consistent with infection by inhalation.

Few outbreaks of tularemia caused by airborne transmission of the bacteria have been reported. These outbreaks include a notable outbreak of inhalational tularemia in Sweden in 1966–1967 associated with environmental exposure of farmers in which >600 cases were diagnosed (*28*).There were clusters of outbreaks on Martha's Vineyard (Massachusetts, USA) in 1978 and 2000 (*29,30*) and cases of tularemia caused by airborne transmission to 53 farmers in northern Finland during 1982. (*31*). In Germany in 2005, a total of 39 participants in a hare hunt were infected after exposure to contaminated droplets generated by rinsing infected hares (*32*).

Isolates from Spain obtained during the second tularemia outbreak had the same genotypes as those obtained during the first outbreak (Figures 1, 4). Furthermore, we did not observe any relationships between genotype and geographic origin, or host from which the isolates were recovered, which suggested that in that area, the same clones were circulating in all hosts (Figure 3, panel B). These results are useful because the 2 outbreaks had substantial epidemiologic differences (*10,12*). Our findings indicate that the outbreak in 2007, after 10 years of no epidemiologic activity, was not caused by introduction of a new strain, but by reemergence of an endemic bacterial population that has been circulating in the region for at least the past 15 years. Furthermore, our findings also suggest that clinical forms of the outbreak are determined by ecologic processes involved in infection (e.g., route of infection, infective dose) rather than by the genotype of the pathogen.

In conclusion, we report genetic characterization of *F. tularensis* subsp. *holarctica* isolated in Spain during 2 of the largest tularemia outbreaks worldwide. There were marked epidemiologic differences between the 2 outbreaks, which were separated by 10 years of no epidemic activity. Molecular investigations showed that both outbreaks were caused

by the same group of closely related genotypes in subclade B.Br:FTNF002–00. Therefore, the reemergence of tularemia in 2007 was presumably not caused by introduction of a new strain, but by persistence of local reservoirs of infection. These findings, along with sporadic cases of tularemia in 1998 and 2007, suggest that local foci of tularemia have become established in Spain. Further investigations will help identify these endemic foci and clarify biotic and abiotic factors that have favored establishment of the pathogen in northwestern Spain.

This study was supported by project PEP 2009/1422 of the Junta de Castilla y León (Spain). A.J. was supported by the Laboratory for Molecular Infection Medicine Sweden within the Nordic European Molecular Biology Laboratory Partnership for Molecular Medicine and by the Västerbotten County Council.

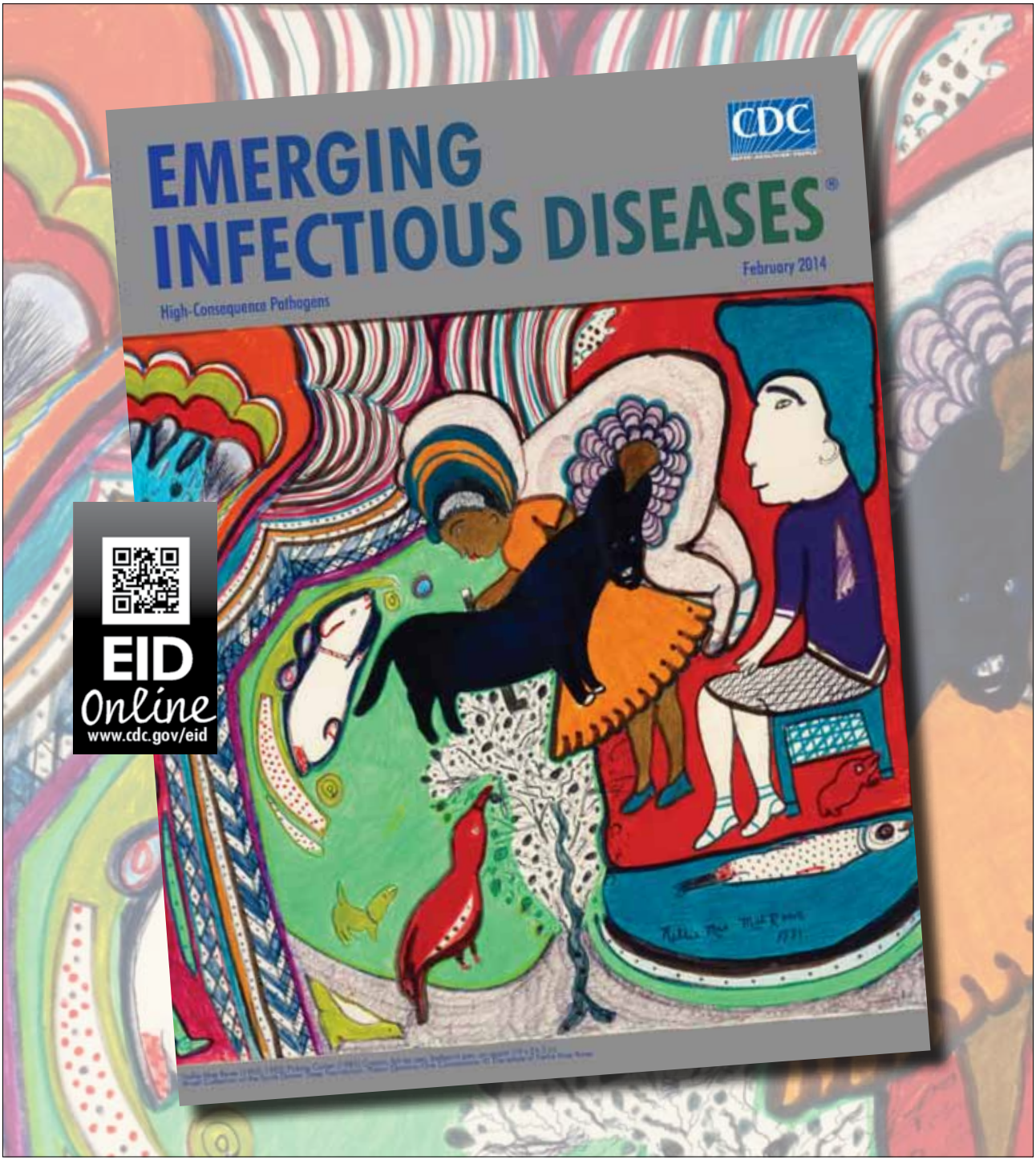
Dr Ariza-Miguel is a biologist and PhD candidate at the Instituto Tecnológico Agrario de Castilla y León, Valladolid, Spain. His primary research interest is emerging infectious diseases.

References

1. Hopla CE, Hopla AK. Tularemia. In: Beran GW, Steele JH, editors. Handbook of zoonoses. 2nd ed. Boca Raton (FL): CRC Press, Inc.; 1994. p. 113–26.
2. Possession, use, and transfer of select agents and toxins; biennial review. Department of Health and Human Services [cited 2013Apr 23]. http://www.gpo.gov/fdsys/pkg/FR-2012-10-05/html/2012-24389.htm
3. Sjöstedt AB. Family XVII. Francisellaceae, genus I. *Francisella*. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM, editors. Bergey's manual of systematic bacteriology. 2nd ed., vol. 2 (The proteobacteria), part B (The gammaproteobacteria). New York: Springer; 2005. p. 200–10.
4. Gurycová D. First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. Eur J Epidemiol. 1998;14:797–802. http://dx.doi.org/10.1023/A:1007537405242
5. Johansson A, Farlow J, Larsson P, Dukerich M, Chambers E, Byström M, et al. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J Bacteriol. 2004;186:5808–18. http://dx.doi.org/10.1128/JB.186.17.5808-5818.2004
6. Larsson P, Svensson K, Karlsson L, Guala D, Granberg M, Forsman M, et al. Canonical insertion-deletion markers for rapid DNA typing of *Francisella tularensis*. Emerg Infect Dis. 2007;13:1725–32. http://dx.doi.org/10.3201/eid1311.070603
7. Vogler AJ, Birdsell D, Price LB, Bowers JR, Beckstrom-Sternberg SM, Auerbach RK, et al. Phylogeography of *Francisella tularensis*: global expansion of a highly fit clone. J Bacteriol. 2009;191:2474–84. http://dx.doi.org/10.1128/JB.01786-08
8. Ellis J, Oyston PC, Green M, Titball RW. Tularemia. Clin Microbiol Rev. 2002;15:631–46. http://dx.doi.org/10.1128/CMR.15.4.631-646.2002
9. Keim PS, Wagner DM. Humans, evolutionary and ecologic forces shaped the phylogeography of recently emerged diseases. Nat Rev Microbiol. 2009;7:813–21. http://dx.doi.org/10.1038/nrmicro2219
10. De Mateo S, Coisin CR. Outbreak of tularaemia in Castilla y León, Spain. Euro Surveill. 1998. [cited 2013]. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18948
11. Avery FW, Barnett TB. Pulmonary tularemia: a report of five cases and consideration of pathogenesis and terminology. Am Rev Respir Dis. 1967;95:584–91.
12. Martín C, Gallardo MT, Mateos L, Vian E, Garcia MJ, Ramos J, et al. Outbreak of tularaemia in Castilla y León, Spain. Euro Surveill. 2007;12:E071108.1.
13. Allue M, Sopeña CR, Gallardo MT, Mateos L, Vian E, Garcia MJ, et al. Tularaemia outbreak in Castilla y León, Spain, 2007: an update. Euro Surveill. 2008;13:18948.
14. Anda P, Segura del Pozo J, Díaz García JM, Escudero R, García Peña FJ, López Velasco MC, et al. Waterborne outbreak of tularemia associated with crayfish fishing. Emerg Infect Dis. 2001;7:575–82.
15. Broekhuijsen M, Larsson P, Johansson A, Byström M, Eriksson U, Larsson E, et al. Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. J Clin Microbiol. 2003;41:2924–31. http://dx.doi.org/10.1128/JCM.41.7.2924-2931.2003
16. Emanuel PA, Bell R, Dang JL, McClanahan R, David JC, Burgess RJ, et al. Detection of *Francisella tularensis* within infected mouse tissues by using a hand-held PCR thermocycler. J Clin Microbiol. 2003;41:689–93. http://dx.doi.org/10.1128/JCM.41.2.689-693.2003
17. Dempsey MP, Dobson M, Zhang C, Zhang M, Lion C, Gutiérrez-Martín CB, et al. Genomic deletion marking an emerging subclone of *Francisella tularensis* subsp. *Holarctica* in France and the Iberian Peninsula. Appl Environ Microbiol. 2007;73:7465–70. http://dx.doi.org/10.1128/AEM.00646-07
18. García Del Blanco N, Dobson ME, Vela AI, De La Puente VA, Gutiérrez CB, Hadfield TL, et al. Genotyping of *Francisella tularensis* strains by pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting, and 16S rRNA gene sequencing. J Clin Microbiol. 2002;40:2964–72. http://dx.doi.org/10.1128/JCM.40.8.2964-2972.2002
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. http://dx.doi.org/10.1093/molbev/msm092
20. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol. 1988;26:2465–6.
21. Comparing partitions. Online tool for quantitative assessment of classification agreement [cited 2013 Apr 23]. http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Home
22. Farlow J, Wagner DM, Dukerich M, Stanley M, Chu M, Kubota K, et al. *Francisella tularensis* in the United States. Emerg Infect Dis. 2005;11:1835–41. http://dx.doi.org/10.3201/eid1112.050728
23. Gyuranecz M, Birdsell DN, Splettstoesser W, Seibold E, Beckstrom-Sternberg SM, Makrai L, et al. Phylogeography of *Francisella tularensis* subsp. *holarctica*, Europe. Emerg Infect Dis. 2012;18:290–3. http://dx.doi.org/10.3201/eid1802.111305
24. Vogler AJ, Birdsell DN, Lee J, Vaissaire J, Doujet CL, Lapalus M, et al. Phylogeography of *Francisella tularensis* ssp. *holarctica* in France. Lett Appl Microbiol. 2011;52:177–80. http://dx.doi.org/10.1111/j.1472-765X.2010.02977.x
25. Pilo P, Johansson A, Frey J. Identification of *Francisella tularensis* cluster in central and western Europe. Emerg Infect Dis. 2009;15:2049–51. http://dx.doi.org/10.3201/eid1512.080805
26. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233–9.
27. Ho SY, Lanfear R, Bromham L, Phillips MJ, Ssoubrier J, Rodrigo AG, et al. Time-dependent rates of molecular evolution. Mol Ecol. 2011;20:3087–101. http://dx.doi.org/10.1111/j.1365-294X.2011.05178.x
28. Dahlstrand S, Ringertz O, Zetterberg B. Airborne tularemia in Sweden. Scand J Infect Dis. 1971;3:7–16.

29. Feldman KA, Enscore RE, Lathrop SL, Matyas BT, McGuill M, Schriefer ME, et al. An outbreak of primary pneumonic tularemia on Martha's Vineyard. N Engl J Med. 2001;345:1601–6. http://dx.doi.org/10.1056/NEJMoa011374
30. Teutsch SM, Martone WJ, Brink EW, Potter ME, Eliot G, Hoxsie R, et al. Pneumonic tularemia on Martha's Vineyard. N Engl J Med. 1979;301:826–8. http://dx.doi.org/10.1056/NEJM 197910113011507
31. Syrjälä H, Kujala P, Myllylä V, Salminen A. Airborne transmission of tularemia in farmers. Scand J Infect Dis. 1985;17:371–5.
32. Hauri AM, Hofstetter I, Seibold E, Kaysser P, Eckert J, Neubauer H, et al. Investigating an airborne tularemia outbreak, Germany. Emerg Infect Dis. 2010;16:238–43. http://dx.doi.org/10.3201/eid1602.081727

Address for correspondence: David Rodríguez-Lázaro, Instituto Tecnológico Agrario de Castilla y León, Carretera de Burgos Km. 119, CP 47071, Valladolid, Spain; email: ita-rodlazda@itacyl.es



Streptococcus mitis Strains Causing Severe Clinical Disease in Cancer Patients

Samuel A. Shelburne, Pranoti Sahasrabhojane, Miguel Saldana, Hui Yao, Xiaoping Su, Nicola Horstmann, Erika Thompson, and Anthony R. Flores

The genetically diverse viridans group streptococci (VGS) are increasingly recognized as the cause of a variety of human diseases. We used a recently developed multilocus sequence analysis scheme to define the species of 118 unique VGS strains causing bacteremia in patients with cancer; *Streptococcus mitis* (68 patients) and *S. oralis* (22 patients) were the most frequently identified strains. Compared with patients infected with non–*S. mitis* strains, patients infected with *S. mitis* strains were more likely to have moderate or severe clinical disease (e.g., VGS shock syndrome). Combined with the sequence data, whole-genome analyses showed that *S. mitis* strains may more precisely be considered as ≥2 species. Furthermore, we found that multiple *S. mitis* strains induced disease in neutropenic mice in a dose-dependent fashion. Our data define the prominent clinical effect of the group of organisms currently classified as *S. mitis* and lay the groundwork for increased understanding of this understudied pathogen.

Viridans group streptococci (VGS), a genetically heterogeneous group of bacteria, are the predominant bacteria in the human oropharynx (*1*). VGS cause a wide range of infections in humans, including bacteremia in patients with neutropenia, infective endocarditis, and orbital cellulitis (*2*–*5*). However, despite the substantial clinical effect of VGS, the epidemiology and pathogenesis of these bacteria are minimally understood (*6*).

A major impediment to the study of VGS has been the inability to consistently and accurately assign VGS strains to specific species, which has resulted in numerous changes in species designation and classification schemes over time (*7*). From a clinical microbiology laboratory standpoint, automated systems have considerable limitations in VGS species identification (*8*,*9*). The problematic nature of VGS species assignment also extends to16S rRNA sequencing, the most widely used genetic tool for species identification in clinical and research settings (*9*,*10*).

Outcomes for patients with VGS bacteremia are highly variable: some patients have minimal symptoms, and others have a severe infection characterized by hypotension and acute respiratory distress syndrome (*11*). The severe infections have been termed VGS shock syndrome (*12*). Numerous studies have examined the species distribution of VGS that cause bacteremia (*9*,*13*–*16*). However, these studies have found inconsistent results between a particular VGS species and disease occurrence or clinical severity of infection (*9*,*13*,*14*,*16*,*17*). Moreover, the recently recognized limitations of previously used techniques of VGS species identification and the low number of clinical cases analyzed preclude definitive conclusions regarding the relationship between VGS species type and clinical disease (*8*,*9*,*18*). Thus, we sought to combine the species identification of a large number of VGS bloodstream isolates, which we typed by using a recently developed multilocus sequence analysis (MLSA) technique (*19*), with patient-specific clinical data to determine relationships between VGS species and clinical endpoints.

Materials and Methods

Study Cohort and Data Abstraction

The study cohort comprised patients at MD Anderson Cancer (MDACC) who had VGS isolated from their blood between July 1, 2011, and December 1, 2012. MDAAC is a 600-bed referral cancer hospital in Houston, Texas, USA.

Author affiliations: MD Anderson Cancer Center, Houston, Texas, USA (S.A. Shelburne, P. Sahasrabhojane, M. Saldana, H. Yao, X. Su, N. Horstmann, E. Thompson); and Baylor College of Medicine, Houston, Texas, USA (A.R. Flores)

DOI: http://dx.doi.org/10.3201/eid2005.130953

We used a standardized data collection form to abstract clinical data from the comprehensive electronic medical records of patients with blood culture results positive for VGS. Antimicrobial drug resistance was determined in accordance with guidelines of the Clinical and Laboratory Standards Institute (www.clsi.org/standards/). VGS are known to contaminate blood cultures and to cause clinically minor, transient bacteremia, and differentiating between contamination and infection is problematic (*20*). Thus, for the purpose of this study, we considered that patients without signs or symptoms of infection had clinically minor bacteremia, even though they may represent cases of blood culture contamination.

Severity of infection, as measured by the Pitt bacteremia score, was determined as described (*21*). Pitt bacteremia scores were not determined for patients with polymicrobial bacteremia. VGS shock syndrome was defined by using the accepted definition for septic shock (i.e., hypotension refractory to fluid replacement in the setting of an infection) (*22*). A focus of the bloodstream infection was defined as isolation of a VGS species from a nonsterile site (e.g., liver abscess) at the same time that VGS were isolated from the blood, with the exception of infective endocarditis, which was defined according the modified Duke criteria (*23*,*24*). Neutropenia was defined as an absolute neutrophil count of <500 cells/mL.

Some patients had signs and symptoms of a lower respiratory infection and x-ray findings compatible with a pneumonic process that could not be explained (i.e., no known respiratory pathogens were isolated and no other alternative explanation, e.g., congestive heart failure, was found). Such patients were defined as having unexplained pulmonary infiltrates. Because VGS are considered normal flora, isolation of these organisms from a respiratory specimen would not have been considered clinically meaningful by the clinical microbiology laboratory and thus would not have been reported. The study protocol was approved by the MDCC institutional review board.

VGS Species Type Determination and Whole-Genome Sequencing

Bacterial isolates were identified as VGS on the basis of the following: presence of α-hemolysis, gram-positive reaction, coccus morphology arranged in chains, negative catalase test results, and exclusions of pneumococcus and enterococci by routine biochemical tests (i.e., optochin, bile solubility, and pyrrolidonyl arylamidase tests) (*25*). VGS species was determined as described (*19*). In brief, concatenated sequences of 7 housekeeping genes were used for phylogenetic tree construction in MEGA5 (www.megasoftware.net/); strains were assigned to VGS species on the basis of their distance from species type strains (*19*). For whole-genome sequencing of 9 *Streptococcus mitis* strains and 1 *S. oralis* strain, we fragmented 3 μg of genomic DNA to 350 bp (mean fragment size) and prepared barcoded sequencing libraries. The 03/10libraries were sequenced on the HiSeq 2000 sequencing System (Illumina, San Diego, CA, USA) by using 76-bp, paired-end sequencing. The raw reads in FASTQ format were aligned to the *S. mitis* B6 (GenBank accession no. NC_013853.1) and *S. pneumoniae* TIGR4 (GenBank accession no. NC_003028.3) genomes by using Mosaik (*26*). There was an average of 250× coverage per base, indicating extremely high confidence for base calls. Contigs were generated by feeding the raw genome sequence data into the A5 pipeline (*27*). Gene annotations were obtained by uploading contigs to the Rapid Annotation using the Subsystem Technology server at the National Microbial Pathogen Data Resource website (*28*). (Individual gene sequencing data have been deposited into GenBank. Short-read sequencing data have been deposited to the Short Read Archive (www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=announcement)underaccessionno. PRJNA240080.

Mouse Infection Studies

Experiments in mice were performed according to a protocol approved by the MDACC Institutional Animal Care and Use Committee. To induce neutropenia, we injected 5-week-old female Balb/C mice intraperitoneally with 100 μg/kg of cyclophosphamide (Sigma, St. Louis, MO, USA) on days –4 and –1 before bacterial injection. On day 0, mice (10 per bacterial dose) were injected with 100 mL of phosphate-buffered saline (PBS) containing 10-fold increments of bacteria ranging from 10^3 to 10^7 CFUs. As a control, 10 mice were injected with PBS alone. Mice were monitored over 7 days for near-death status. The dose at which 50% of the mice nearly died (hereafter referred to as LD_{50}) was calculated by using the probit method. Neutropenia was confirmed in select mice on postinfection days 1, 3, and 6.

Statistical Analysis

Differences between categorical variables were assessed by using the χ^2 test; Fisher exact test was used when at least 1 category had <5 occurrences. The relationship between VGS species and Pitt bacteremia scores was analyzed by using the Mann-Whitney U test. The Bonferroni method was employed to account for multiple comparisons when appropriate. All tests of significance were 2-sided, and statistical significance was defined at $p \leq 0.05$. SPSS Statistics version 19 (IBM, Armonk, NY, USA) was used for statistical analysis.

Results

Study Cohort

A total of 118 consecutive patients with VGS-positive blood cultures were included in the study cohort; ≈80% of

the patients had neutropenia and hematologic malignancies (Table 1). Most patients had bacteremia without a defined focus, but several other clinical scenarios were observed, including skin/soft tissue infections, gastrointestinal infections, and infective endocarditis. Most patients had clinically mild infections (Pitt bacteremia score of 0 or 1), but 25% of patients had moderate to severe infections (Pitt bacteremia scores of ≥2), including 12 patients who had VGS shock syndrome.

Table 1. Characteristics of 118 participants in a study of *Streptococcus mitis* strains causing severe clinical disease in patients with cancer*

Characteristic	No. (%)
Sex	
M	64 (54)
F	54 (46)
Mean age, y (SD, range)	50 (18, 10–79)
Malignancy	70 (59)
Leukemia/myelodysplastic syndrome	20 (17)
Hematopoietic stem cell transplantation	10 (8)
Lymphoma/myeloma	18 (15)
Solid tumor	20 (17)
Neutrophils <500/μL	95 (81)
Clinical syndrome	
Primary bacteremia	95 (80)
Gastrointestinal focus	8 (7)
Skin/soft tissue focus	4 (3)
Infective endocarditis	2 (2)
Clinically minor bacteremia	9 (7)
Polymicrobial infection	22 (19)
Pitt bacteremia score†‡	
0	35 (37)
1	36 (38)
2	5 (5)
3	7 (7)
≥4	13 (13)
Antimicrobial drug susceptibility	
Penicillin	54 (46)
Ceftriaxone	107 (91)
Moxifloxacin	60 (51)
Tetracycline	69 (59)

*All patients had viridans group streptococci bacteremia. Unless otherwise noted, data are no. (%) of patients.
†Severity of infection was measured by the Pitt bacteremia score as described (*21*). Scores of 0 or 1 indicate clinically mild infections; scores of ≥2 indicate moderate to severe infections.
‡Determined only for patients with monomicrobial infection.

VGS Species and Bacteremia

To gain insight into the species of VGS causing bacteremia in the study cohort, we performed MLSA of 7 housekeeping genes, as described (*19*). Strains were assigned to species by comparing their position on the phylogenetic tree with those of established type strains (*19*). The 118 strains could be confidently assigned to 11 distinct species (Figure 1; online Technical Appendix 1, wwwnc.cdc.gov/EID/article/20/5/13-0953-Techapp1.xlsx). The most commonly observed species were *S. mitis* (68 strains), *S. oralis* (22 strains), and *S. parasanguinis* (12 strains). For classification purposes, various VGS species are often placed into distinct groups; the association between VGS strains causing bacteremia and group assignment is shown in Figure 1, using the scheme from Sinner et al. (*29*). In total, 80% of strains were from the Mitis group, and the remaining strains were from the Sanguinis group (14%), Anginosus group (3%), and Salivarius group (3%).

VGS Species and Clinical Syndromes

Because of the diverse genetic nature of the various VGS species, we next tested the hypothesis that distinct VGS species cause different clinical syndromes. Given the number of strains for each species, we analyzed the Mitis group species (i.e., *S. mitis* and *S. oralis*) individually and analyzed species comprising the Sanguinis, Anginosus, and Salivarius groups by group (Table 2). Compared with strains of other VGS species, *S. mitis* strains were significantly more likely to cause primary bacteremia ($p<0.01$) and less likely to cause polymicrobial bacteremia ($p = 0.01$) and clinically minor bacteremia ($p<0.01$). *S. oralis* strains were more likely to cause polymicrobial infection ($p = 0.02$), Sanguinis group strains were more likely to cause clinically minor bacteremia ($p<0.01$), and Anginosus group strains were significantly associated with bacteremia with a gastrointestinal focus ($p<0.01$). When we only considered patients with neutropenia or cases of monomicrobial bacteremia, we observed the same statistically significant species–clinical disease relationships (data not shown).

VGS Species and Disease Severity

We next sought to determine if there was a relationship between VGS species and disease severity (as determined by Pitt bacteremia score). Organ dysfunction, such as hypotension, begins to occur at Pitt bacteremia scores of ≥2 (*21*). The distribution of Pitt bacteremia score by infecting species is shown in Figure 2, panel A. Patients infected with *S. mitis* were significantly more likely to have a higher Pitt bacteremia score ($p<0.01$). One possible explanation for this observation is that *S. mitis* strains mainly caused infections in patients with neutropenia who, compared with patients without neutropenia, might be more likely to have serious infections. Thus, we repeated the analysis, including only patients with neutropenia. Again, the Pitt bacteremia scores were significantly higher for patients infected with *S. mitis* ($p<0.01$; Figure 2, panel B). Of the 12 cases of VGS shock syndrome, 11 were caused by *S. mitis* strains and 1 was caused by an *S. constellatus* strain (Anginosus group).

Identification of *S. mitis* Strain Clusters

Most cases of bacteremia and severe disease occurred in patients infected with *S. mitis*; thus, we focused on *S. mitis* strains and strains from the closely related *S. oralis* species. In contrast to what we observed for the *S. oralis* strains, several distinct groupings could be visualized within the *S. mitis* strains, which we arbitrarily labeled as

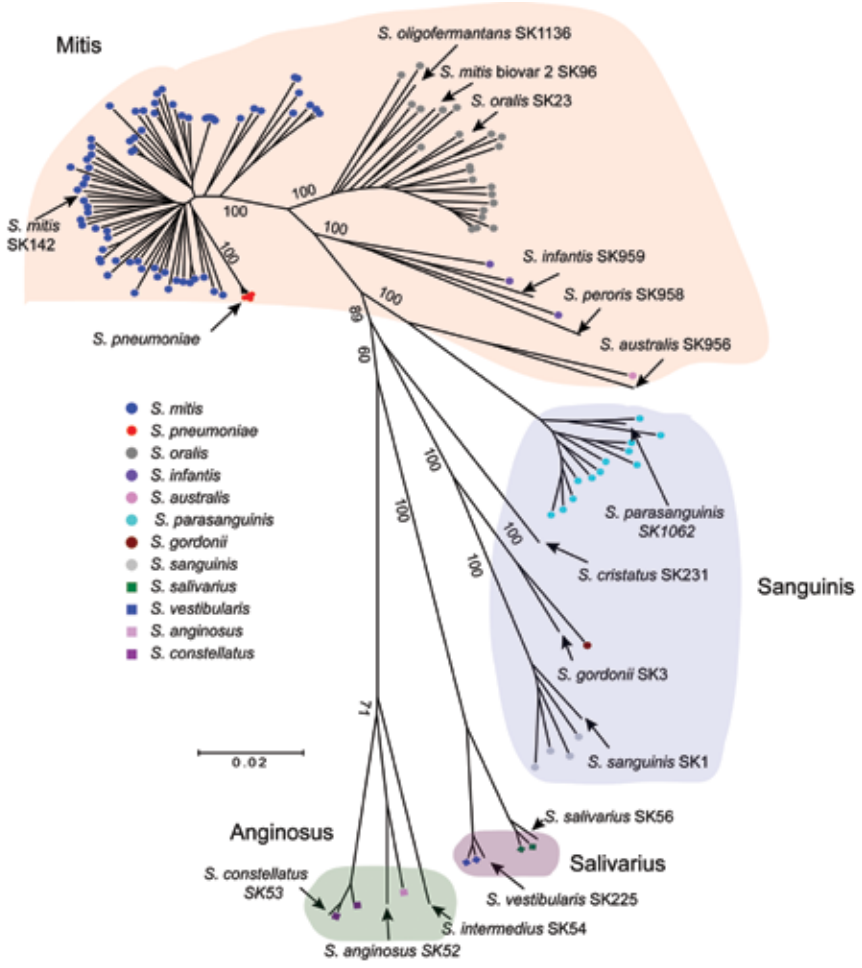


Figure 1. Multilocus sequence analysis (MLSA) of viridans group streptococci (VGS) strains causing bacteremia in patients with cancer. The neighbor-joining radial tree was generated by using concatenated sequences. Strains were assigned to a particular VGS on the basis of their proximity to type strains. Locations of well-characterized or type VGS strains (lines without circles) are also shown for reference purposes. Five contemporaneous *Streptococcus pneumoniae* strains are also included for reference purposes (shown in red). Numbers indicate bootstrap support values (%).Scale bar indicates genetic distance. Background colors indicate VGS species group, using the system from Sinner et al. (*29*).

clusters 1, 2, and 3 (Figure 3, panel A). *S. mitis* cluster 1 comprised 10 strains, including 2 that were genetically identical by MLSA, and cluster 2 comprised 22 strains, including 6 that were genetically identical. Cluster 3 comprised 29 strains and may contain additional strain groupings, but further phylogenetic delineation of this cluster could not be done with sufficient confidence. We did not observe substantial differences, in terms of distinct disease types or severity of infection, between patients from whom the *S. mitis* cluster strains were derived (online Technical Appendix 2 Figure, wwwnc.cdc.gov/EID/article/20/5/13-0953-Techapp1.pdf). However, there was a predominance of unexplained pulmonary infiltrate cases among patients infected with cluster 2 strains ($p<0.01$ for cluster 2 strains vs. noncluster 2 strains) (Figure 3, panel B). This variable was investigated because, given the close genetic relationship between *S. mitis* and *S. pneumoniae*, we hypothesize that *S. mitis* strains may cause pneumonia in severely immunocompromised persons (Figure 1).

Whole-Genome Analysis and MLSA Grouping of Strains

To determine whether the MLSA data accurately represented the entire genetic content of the Mitis group

Table 2. Association between clinical syndrome and infecting species for 118 patients with viridans group streptococci bacteremia

	Viridans group streptococci*					
Clinical syndrome	*mitis*	*oralis*	*infantis/australis*	Sanguinis	Anginosus	Salivarius/Vestibularis
Primary bacteremia with neutropenia	58	6	3	3	0	2
Primary bacteremia without neutropenia	1	2	0	3	0	0
Gastrointestinal focus	1	2	0	1	3	1
Skin/soft tissue focus	2	1	0	1	0	0
Infective endocarditis	0	2	0	0	0	0
Polymicrobial bacteremia	6	8	0	4	0	0
Clinically minor bacteremia	0	1	1	5	0	2

**mitis, oralis*,*infantis*, and a*ustralis* refer to viridans group streptococci species; Sanguinis, Anginosus, Salivarius, and Vestibularis refer to viridans streptococci groups (Figure 1) (*29*).

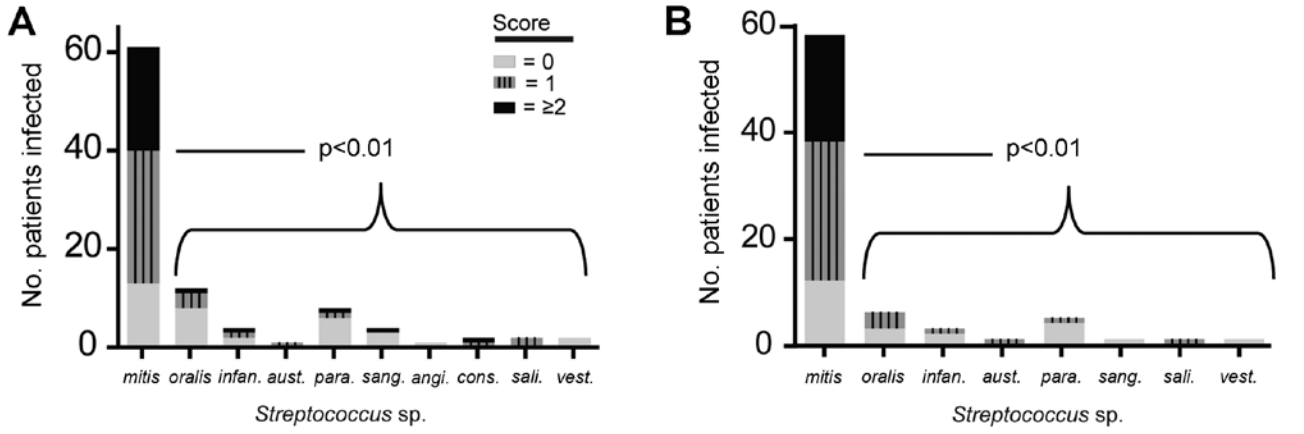


Figure 2. A) Pitt bacteremia scores for cancer patients infected with particular VGS species, showing that more clinically severe disease is caused by *Streptococcus mitis* strains than other viridans group streptococci (VGS) species. B) Pitt bacteremia scores for only those cancer patients with neutropenia. p values refer to Mann-Whitney U comparison of Pitt bacteremia scores for patients infected with *S. mitis* strains versus those infected with non–*S. mitis* strains. *infan.*, *infantis*; *aust.*, *australis*; *para.*, p*arasanguinis; sang.*, *sanguinus*; *angi.*, *anginosus*; *cons.*, *constellatus*; *sali.*, *salivarius*; *vest.*, *vestibularis*.

strains, we performed whole-genome sequencing of 9 *S. mitis* and 1 *S. oralis* isolates (Figure 4, panel A). For the 9 *S. mitis* strains, the reads mapped to ≈70% coverage of the only completely finished *S. mitis* genome (*S. mitis* strain B6 [*30*]), Technical Appendix 2, Table 1). This considerable level of intraspecies genetic diversity for *S. mitis* strains has been observed previously in sequencing and DNA:DNA hybridization studies and meant that we could not use whole-genome analysis of single-nucleotide polymorphisms to determine strain relatedness (*30*,*31*). Thus, we next sought to identify regions of genetic similarity among the strains that could be analyzed for interstrain comparisons.

All 9 *S. mitis* strains contained operons encoding a putative polysaccharide capsule. The first 4 genes of the operon, corresponding to *cpsA–cpsD* in *S. pneumoniae,*

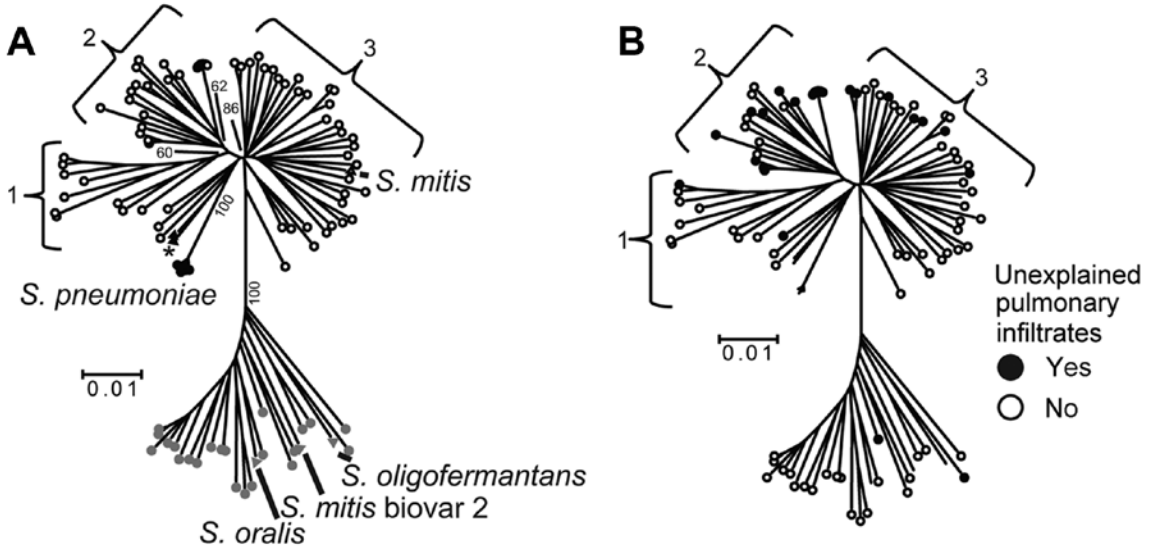


Figure 3. Multilocus sequence analysis and clinical correlates of Streptococcus mitis and S. oralis strains. A) Open circles are S. mitis strains from this study; gray circles are S. oralis strains from this study. For reference purposes, the following are labeled: viridans group streptococci (VGS) strains (SK142 for S. mitis, SK23 for S. oralis, SK1136 for S. oligofermantans), 5 S. pneumoniae strains (black circles), 2 S. pseudopneumoniae strains (SK674 and 103, indicated by an asterisk), and strain SK96 (previously characterized as an S. mitis biovar 2 strain). Numbers within the tree refer to bootstrap support values (%). A, B) Numbers 1–3 indicate S. mitis clusters, and scale bars indicate genetic distances. B) For reference purposes, branches of the previously labeled VGS, S. pneumoniae, and S. pseudopneumoniae strains have been retained; however, for clarity, the branches are not labeled. The presence or absence of unexplained pulmonary infiltrates is indicated as described in the key. Bootstrap support values are the same as in panel A.

were relatively well conserved among the 9 strains. Concatenated alignment of *cpsA–cpsD* showed a close relationship for the 4 cluster 2 strains and strain Shelburne VGS (SVGS) 003, whereas the *cpsA–cpsD* genes from the remaining 4 strains were more closely related to the *S. pneumoniae* strain TIGR4 (SVGS004 and SVGS019) or to the *S. mitis* type strain SK142 (SVGS002 and SVGS011) (Figure 4, panel B).

In addition to the capsule operons, multiple other comparisons arising from our whole-genome analysis confirmed the idea that the 4 cluster 2 strains and strain SVGS003 were closely related. All of the *S. mitis* strains

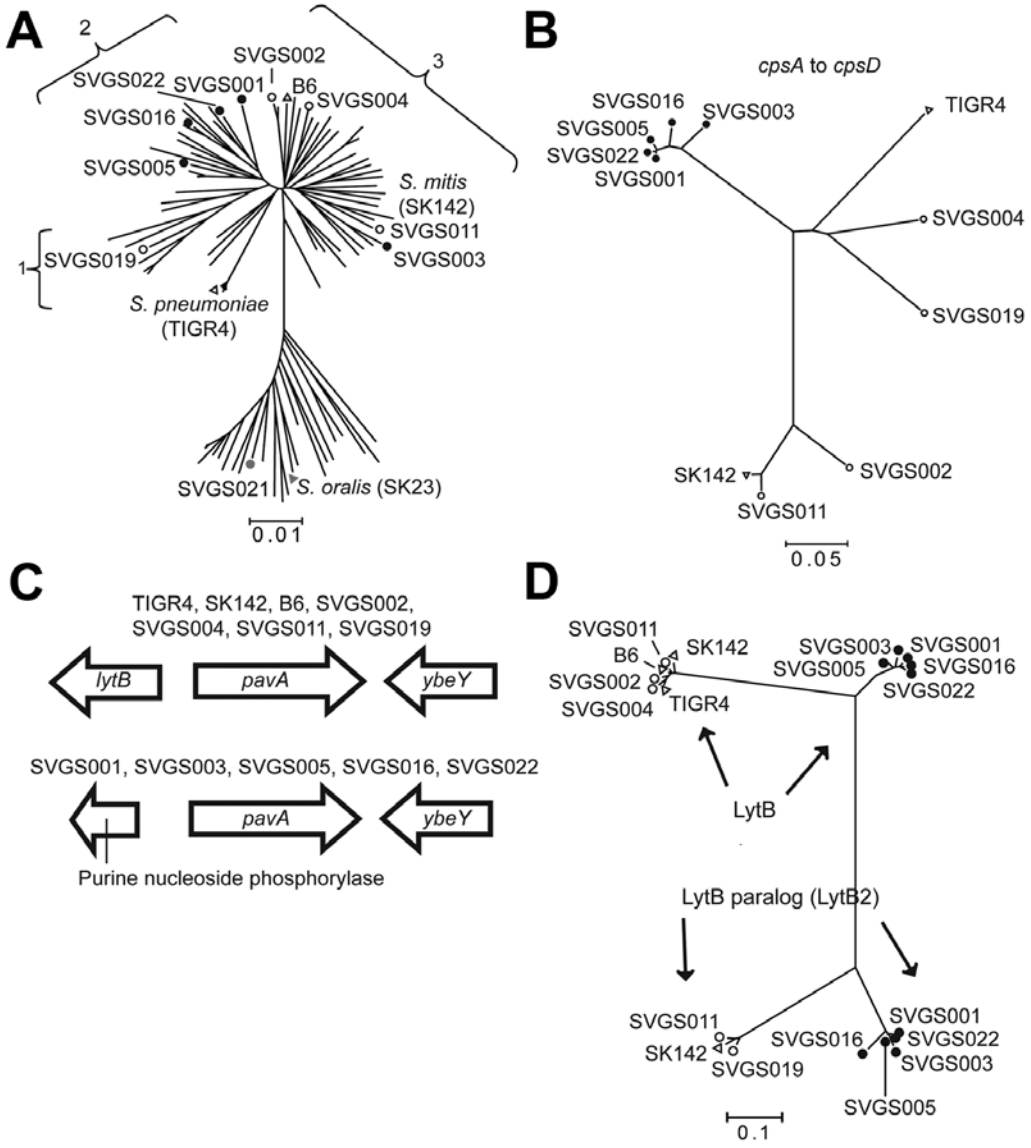


Figure 4. Selected data from whole-genome analysis of viridans group streptococci (VGS) strains. A) Neighbor-joining tree generated by multilocus sequence analysis (MLSA) of *Streptococcus mitis* and *S. oralis* strains, showing locations of VGS strains selected for whole-genome analysis. Numbers 1–3 refer to *S. mitis* clusters (defined in Figure 3). MLSA locations are also shown for the *S. mitis* and *S. oralis* type strains (SK142 and SK23, respectively) and fully sequenced *S. mitis* strain B6 and *S. pneumoniae* strain TIGR4. B) SVGS004 mouse challenge data. Neighbor-joining tree of first 4 genes of the capsular polysaccharide encoding operon (*cpsA–cpsD*). TIGR4 and SK142 are included for reference purposes. Strain B6 is not included because it lacks a *cps* operon. Note tight clustering of 5 VGS strains (black dots). C) Genetic arrangement surrounding the *pavA* gene, which encodes a fibronectin-binding protein. Two distinct gene arrangements are present 5′ of the *pavA* gene, with the arrangement for particular strains as indicated. D) Neighbor-joining tree of LytB protein, which is involved in cell-wall turnover, from fully sequenced *S. mitis* strains. Some *S. mitis* strains possess a gene encoding a second LytB-like protein, which we have named LytB2 (ZP_07643922 from strain SK142). Note tight clustering of the same 5 VGS strains (black dots) for the LytB and LytB2 proteins as was observed for the *cpsA–cpsD* analysis in panel B. A, B, D) SVGS, Shelburne VGS.Scale bars indicate genetic distances.

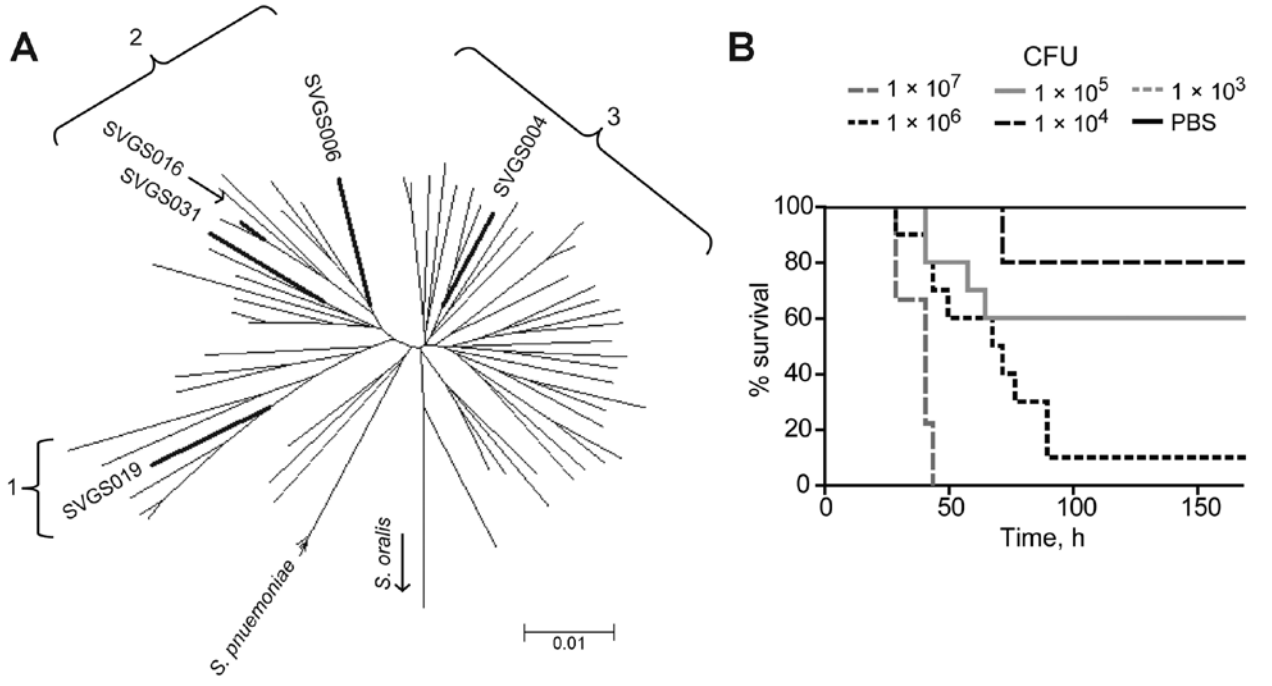


Figure 5. *Streptococcus mitis* strains cause dose-dependent disease in mice with neutropenia. A) Multilocus sequence analysis–generated neighbor-joining tree showing genetic relationships among all *S. mitis* strains. Bold branches indicate locations of the 5 strains used in the mouse model challenge experiment. Numbers 1–3 refer to clusters of *S. mitis* strains (defined in Figure 3). Scale bar indicates genetic distance. B) Example of mouse challenge data. Ten neutropenic Balb/C mice per dose were infected intraperitoneally with serial 10-fold CFU dilutions of strain SVGS004 (range 10^7–10^3) and monitored for 168 h (7 d) for near-death status. Phosphate-buffered saline (PBS) was injected as a control. None of the mice injected with PBS or with the 10^3 dose were near death; thus, the 1×10^3 line is obscured by the PBS line on the graph. SVGS, Shelburne viridans group streptococcus.

contained the gene encoding the fibrinogen-binding protein, PavA (pneumococcal adherence and virulence protein A). However, there was a different gene 5′ to the *pavA* gene in SVGS003 and the 4 cluster 2 strains than in the other 4 *S. mitis* strains (Figure 4, panel C). In a similar manner, the LytB protein in cluster 2 strains and SVGS003 grouped separately from the LytB protein in the other strains, and a LytB paralog in strain SVGS003 and the 4 cluster 2 strains was distinct from other forms of the LytB protein and from LytB paralogs encoded by SVGS011 and SVGS019.

LytB is part of a group of choline-binding proteins that are involved in cell-wall turnover, some of which have been shown to be important for virulence in *S. pneumoniae* (*32*). When the presence or absence of choline-binding proteins was determined for the various strains, substantial strain-to-strain heterogeneity was observed (Technical Appendix 2 Table 2). The only repeating pattern of gene content was 1 that occurred for 3 different choline-binding protein–encoding genes: *cbpE, cbpI,* and *lytC*. These 3 genes, which are present in diverse chromosomal locations, were absent in the 4 cluster 2 strains, SVGS003, and the *S. oralis* strain SVGS021 but present in the other noncluster 2 *S. mitis* strains. Thus, the whole-genome data support MLSA data, assigning 4 of the fully sequenced strain to *S. mitis* cluster 2; strain SVGS003, a group 3 strain by MLSA, appears to have genetic characteristics of the cluster 2 strains by whole-genome analysis.

Mouse Model for Testing VGS Virulence

Given the apparent differences in genetic content among *S. mitis* strains, we sought to develop an animal model for testing VGS virulence that would approximate the disease observed in cancer patients with neutropenia. No neutropenia model of VGS infection exists, so we used serial 10-fold CFU dilutions of 5 *S. mitis* strains to determine the LD_{50} of organisms for the endpoint of being near death. The *S. mitis* challenge strains included isolates from the major *S. mitis* clusters (Figure 5; Table 3), and we also injected PBS as a control. None of the mice injected with PBS became ill, indicating that neither the neutropenia nor the injection itself caused major disease (Figure 5, panel B). All of the *S. mitis* strains could cause near-death status, and a dose–response relationship was observed for all strains (see example in Figure 5, panel B), but the LD_{50} varied by 100-fold among the strains (Table 3). Strain SVGS016, which caused the most severe clinical disease (i.e., it was isolated from a patient with the highest Pitt bacteremia score) also was the most virulent in the mouse model. Thus,

Table 3. Relative virulence of viridans group streptococci strains in a neutropenic mouse model*

Strain	*Streptococcus mitis* cluster	Pitt bacteremia score of infected patient	LD_{50}
SVGS004	3	1	4.1×10^5
SVGS006	2	2	1.6×10^6
SVGS016	2	1	1.4×10^5
SVGS019	1	3	1.9×10^4
SVGS031	2	1	3.6×10^5

*SVGS, Shelburne viridans group streptococcus; LD_{50}, considered, for the purpose of this study, to be the dose at which 50% of the animals nearly died.

we suggest that *S. mitis* strains cause disease in mice with neutropenia and that there is differential virulence in this mouse model among genetically diverse *S. mitis* strains.

Conclusions

Since first being identified as causative agents of infections in cancer patients with neutropenia ≈35 years ago (*33*), VGS have come to be appreciated as major bacterial pathogens in patients with malignancy (*2*,*12*,*14*,*34*,*35*). The emergence of VGS as common infectious agents has coincided with the increasing use of prophylactic antimicrobial drugs, especially fluoroquinolones, for patients with neutropenia (*36*). However, despite the clear clinical consequences of VGS infections, there is minimal understanding of their pathophysiology.

A critical first step in the study of VGS is to define the clinical syndromes caused by various VGS species. This goal has long been hampered by difficulties in using phenotypic methods or single-gene sequencing approaches to assign VGS strains to particular species (*18*). Through the use of a recently developed MLSA approach (*19*), we showed that there is a relationship between VGS species, as defined genetically, and disease manifestations in patients with cancer (Table 2). An unexpected finding was the relationship between Sanguinis group strains and clinically minor bacteremia; Sanguinis group species are the leading VGS cause of infective endocarditis and have been reported to cause virulent infections in patients with neutropenia (*17*,*37*). One possible explanation for this finding is that Sanguinis group VGS are often causative agents of transient bacteremia and that transient bacteremia occasionally results in infective endocarditis. Platelets are thought to be critical to the pathogenesis of VGS infective endocarditis. Thus, because of low platelet counts, persons with cancer, especially those with hematologic malignancy, may be relatively resistant to the development of infective endocarditis after transient VGS bacteremia (*38*).

Another key relationship that we observed was that of *S. mitis* and primary bacteremia during periods of neutropenia. Our data support and extend the findings of other smaller studies using genetic techniques that found a similar predominance of *S. mitis* strains in patients with neutropenia (*9*,*15*,*39*). The reason that *S. mitis* strains are the leading cause of VGS bacteremia in patients with neutropenia is not known. One could postulate that *S. mitis* is simply the dominant commensal VGS species and thus is the most likely species to translocate across epithelial barriers when patients become neutropenic. Indeed, a recent microbiome study showed that *S. mitis* is the predominant VGS species isolated from buccal mucosa samples from healthy persons (*1*). However, in our study, *S. mitis* not only caused the majority of neutropenic infections but also caused a disproportionate percentage of serious infections (Figure 2). Thus, at least for patients with neutropenia, *S. mitis* is more likely than other VGS to enter into the bloodstream and to cause serious infections once there. Moreover, compared with other VGS species, *S. mitis* rarely caused clinically minor bacteremia or polymicrobial infection, suggesting that *S. mitis* strains have inherently virulent properties compared with other VGS. The data from our multistrain, whole-genome sequencing and the development of an animal model of neutropenia and *S. mitis* infection should provide a key platform for elucidating *S. mitis* virulence.

The deep branching pattern produced by MLSA of our *S. mitis* strains isolated from human blood has been observed in other investigations (*19*,*30*) and suggests that the organisms currently grouped as *S. mitis* may more precisely be considered as ≥2 species. The application of whole-genome sequencing to large numbers of *S. mitis* strains will be necessary to fully resolve *S. mitis* strain clusters, as shown by the somewhat discordant results of our MLSA and whole-genome analysis. In addition, we were intrigued by the association of cluster 2 *S. mitis* strains and unexplained pneumonia (Figure 3, panel B). Given the close genetic relationship between *S. mitis* and *S. pneumoniae*, it might be expected that some *S. mitis* strains could cause pneumonia, especially in severely immunocompromised patients. Whether particular subspecies of *S. mitis* can cause pneumonia is an active area of investigation in our laboratory, and if it does, that could help explain the stubbornly low number of pathogens that can be identified for patients with pneumonic syndromes (*40*).

This large series of invasive VGS strains includes detailed molecular and clinical information. By combining these 2 sets of data, we have definitively established the critical role of *S. mitis* strains in invasive VGS infection in patients with cancer and have laid the groundwork for future insights into how these organisms cause serious disease in vulnerable hosts.

Acknowledgment

We thank the clinical microbiology laboratory staff at MD Anderson Cancer Center for their efforts in identifying and saving VGS isolates and Nathaniel Albert and Dimitrios Kontoyiannis for assistance with the neutropenic mouse model.

This study was supported by an internal grant (to S.A.S.) from MD Anderson Cancer Center. H.Y. is supported by the H. A. and Mary K. Chapman Foundation.

Dr Shelburne is an associate professor and physician–scientist at MD Anderson Cancer Center. His research interest is in the influences of bacterial genetics on the clinical signs and symptoms of infectious diseases in humans.

References

1. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486:207–14. http://dx.doi.org/10.1038/nature11234
2. Tunkel AR, Sepkowitz KA. Infections caused by viridans streptococci in patients with neutropenia. Clin Infect Dis. 2002;34:1524–9. http://dx.doi.org/10.1086/340402
3. Dix D, Cellot S, Price V, Gillmeister B, Ethier MC, Johnston DL, et al. Association between corticosteroids and infection, sepsis, and infectious death in pediatric acute myeloid leukemia (AML): results from the Canadian Infections in AML Research Group. Clin Infect Dis. 2012;55:1608–14. http://dx.doi.org/10.1093/cid/cis774
4. Murdoch DR, Corey GR, Hoen B, Miro JM, Fowler VG Jr, Bayer AS, et al. Clinical presentation, etiology, and outcome of infective endocarditis in the 21st century: the International Collaboration on Endocarditis–Prospective Cohort Study. Arch Intern Med. 2009;169:463–73. http://dx.doi.org/10.1001/archinternmed.2008.603
5. Seltz LB, Smith J, Durairaj VD, Enzenauer R, Todd J. Microbiology and antibiotic management of orbital cellulitis. Pediatrics. 2011;127:e566–72. http://dx.doi.org/10.1542/peds.2010-2117
6. Seo HS, Xiong YQ, Mitchell J, Seepersaud R, Bayer AS, Sullam PM. Bacteriophage lysin mediates the binding of *Streptococcus mitis* to human platelets through interaction with fibrinogen. PLoS Pathog. 2010;6:e1001047. http://dx.doi.org/10.1371/journal.ppat.1001047
7. Facklam R. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev. 2002;15:613–30. http://dx.doi.org/10.1128/CMR.15.4.613-630.2002
8. Teles C, Smith A, Ramage G, Lang S. Identification of clinically relevant viridans group streptococci by phenotypic and genotypic analysis. Eur J Clin Microbiol Infect Dis. 2011;30:243–50. http://dx.doi.org/10.1007/s10096-010-1076-y
9. Hoshino T, Fujiwara T, Kilian M. Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. J Clin Microbiol. 2005;43:6073–85. http://dx.doi.org/10.1128/JCM.43.12.6073-6085.2005
10. Kawamura Y, Hou XG, Sultana F, Miura H, Ezaki T. Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. Int J Syst Bacteriol. 1995;45:406–8. http://dx.doi.org/10.1099/00207713-45-2-406
11. Bochud PY, Eggiman P, Calandra T, Van Melle G, Saghafi L, Francioli P. Bacteremia due to viridans streptococcus in neutropenic patients with cancer: clinical spectrum and risk factors. Clin Infect Dis. 1994;18:25–31. http://dx.doi.org/10.1093/clinids/18.1.25
12. Gassas A, Grant R, Richardson S, Dupuis LL, Doyle J, Allen U, et al. Predictors of viridans streptococcal shock syndrome in bacteremic children with cancer and stem-cell transplant recipients. J Clin Oncol. 2004;22:1222–7. http://dx.doi.org/10.1200/JCO.2004.09.108
13. Wisplinghoff H, Reinert RR, Cornely O, Seifert H. Molecular relationships and antimicrobial susceptibilities of viridans group streptococci isolated from blood of neutropenic cancer patients. J Clin Microbiol. 1999;37:1876–80.
14. Elting LS, Bodey GP, Keefe BH. Septicemia and shock syndrome due to viridans streptococci: a case-control study of predisposing factors. Clin Infect Dis. 1992;14:1201–7. http://dx.doi.org/10.1093/clinids/14.6.1201
15. Kitten T, Munro CL, Zollar NQ, Lee SP, Patel RD. Oral streptococcal bacteremia in hospitalized patients: taxonomic identification and clinical characterization. J Clin Microbiol. 2012;50:1039–42. http://dx.doi.org/10.1128/JCM.06438-11
16. Bilgrami S, Feingold JM, Dorsky D, Edwards RL, Clive J, Tutschka PJ. *Streptococcus viridans* bacteremia following autologous peripheral blood stem cell transplantation. Bone Marrow Transplant. 1998;21:591–5. http://dx.doi.org/10.1038/sj.bmt.1701140
17. Husain E, Whitehead S, Castell A, Thomas EE, Speert DP. Viridans streptococci bacteremia in children with malignancy: relevance of species identification and penicillin susceptibility. Pediatr Infect Dis J. 2005;24:563–6. http://dx.doi.org/10.1097/01.inf.0000164708.21464.03
18. Ikryannikova LN, Lapin KN, Malakhova MV, Filimonova AV, Ilina EN, Dubovickaya VA, et al. Misidentification of alpha-hemolytic streptococci by routine tests in clinical practice. Infect Genet Evol. 2011;11:1709–15. http://dx.doi.org/10.1016/j.meegid.2011.07.010
19. Bishop CJ, Aanensen DM, Jordan GE, Kilian M, Hanage WP, Spratt BG. Assigning strains to bacterial species via the Internet. BMC Biol. 2009;7:3. http://dx.doi.org/10.1186/1741-7007-7-3
20. Swenson FJ, Rubin SJ. Clinical significance of viridans streptococci isolated from blood cultures. J Clin Microbiol. 1982;15:725–7.
21. Paterson DL, Ko WC, Von Gottberg A, Mohapatra S, Casellas JM, Goossens H, et al. International prospective study of *Klebsiella pneumoniae* bacteremia: implications of extended-spectrum β-lactamase production in nosocomial Infections. Ann Intern Med. 2004;140:26–32. http://dx.doi.org/10.7326/0003-4819-140-1-200401060-00008
22. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. 1992. Chest. 2009;136(Suppl):e28.
23. Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, et al. Epidemiology of invasive group B streptococcal disease in the United States, 1999–2005. JAMA. 2008;299:2056–65. http://dx.doi.org/10.1001/jama.299.17.2056
24. Li JS, Sexton DJ, Mick N, Nettles R, Fowler VG Jr, Ryan T, et al. Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis. 2000;30:633–8. http://dx.doi.org/10.1086/313753
25. Han XY, Kamana M, Rolston KV. Viridans streptococci isolated by culture from blood of cancer patients: clinical and microbiologic analysis of 50 cases. J Clin Microbiol. 2006;44:160–5. http://dx.doi.org/10.1128/JCM.44.1.160-165.2006
26. Hillier LW, Marth GT, Quinlan AR, Dooling D, Fewell G, Barnett D, et al. Whole-genome sequencing and variant discovery in *C. elegans*. Nat Methods. 2008;5:183–8. http://dx.doi.org/10.1038/nmeth.1179
27. Tritt A, Eisen JA, Facciotti MT, Darling AE. An integrated pipeline for de novo assembly of microbial genomes. PLoS ONE. 2012;7:e42304. http://dx.doi.org/10.1371/journal.pone.0042304
28. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST server: rapid annotations using subsystems technology. BMC Genomics. 2008;9:75. http://dx.doi.org/10.1186/1471-2164-9-75
29. Sinner SW, Tunkel AR. Viridans group streptococci, groups C and G streptococci, and *Gemella morbilliform*. In: Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. 7th ed. Philadelphia: Elsevier; 2011. p. 2434–50.

30. Denapaite D, Bruckner R, Nuhn M, Reichmann P, Henrich B, Maurer P, et al. The genome of *Streptococcus mitis* B6—what is a commensal? PLoS ONE. 2010;5:e9426. http://dx.doi.org/10.1371/journal.pone.0009426
31. Kilian M, Poulsen K, Blomqvist T, Havarstein LS, Bek-Thomsen M, Tettelin H, et al. Evolution of *Streptococcus pneumoniae* and its close commensal relatives. PLoS ONE. 2008;3:e2683. http://dx.doi.org/10.1371/journal.pone.0002683
32. Pérez-Dorado I, Galan-Bartual S, Hermoso JA. Pneumococcal surface proteins: when the whole is greater than the sum of its parts. Mol Oral Microbiol. 2012;27:221–45. http://dx.doi.org/10.1111/j.2041-1014.2012.00655.x
33. Pizzo PA, Ladisch S, Witebsky FG. Alpha-hemolytic streptococci: clinical significance in the cancer patient. Med Pediatr Oncol. 1978;4:367–70. http://dx.doi.org/10.1002/mpo.2950040414
34. Burden AD, Oppenheim BA, Crowther D, Howell A, Morgenstern GR, Scarffe JH, et al. Viridans streptococcal bacteraemia in patients with haematological and solid malignancies. Eur J Cancer. 1991;27:409–11. http://dx.doi.org/10.1016/0277-5379(91)90373-L
35. Villablanca JG, Steiner M, Kersey J, Ramsay NK, Ferrieri P, Haake R, et al. The clinical spectrum of infections with viridans streptococci in bone marrow transplant patients. Bone Marrow Transplant. 1990;5:387–93.
36. Ramphal R. Changes in the etiology of bacteremia in febrile neutropenic patients and the susceptibilities of the currently isolated pathogens. Clin Infect Dis. 2004;39(Suppl 1):S25–31. http://dx.doi.org/10.1086/383048
37. Roberts RB, Krieger AG, Schiller NL, Gross KC. Viridans streptococcal endocarditis: the role of various species, including pyridoxal-dependent streptococci. Rev Infect Dis. 1979;1:955–66. http://dx.doi.org/10.1093/clinids/1.6.955
38. Jung CJ, Yeh CY, Shun CT, Hsu RB, Cheng HW, Lin CS, et al. Platelets enhance biofilm formation and resistance of endocarditis-inducing streptococci on the injured heart valve. J Infect Dis. 2012;205:1066–75. http://dx.doi.org/10.1093/infdis/jis021
39. Westling K, Julander I, Ljungman P, Vondracek M, Wretlind B, Jalal S. Identification of species of viridans group streptococci in clinical blood culture isolates by sequence analysis of the RNase P RNA gene, *rnpB*. J Infect. 2008;56:204–10. http://dx.doi.org/10.1016/j.jinf.2007.12.006
40. Musher DM, Roig IL, Cazares G, Stager CE, Logan N, Safar H. Can an etiologic agent be identified in adults who are hospitalized for community-acquired pneumonia: results of a one-year study. J Infect. 2013;67:11–8. http://dx.doi.org/10.1016/j.jinf.2013.03.003

Address for correspondence: Samuel A. Shelburne, MD, PhD, Department of Infectious Diseases, Department of Genomic Medicine, MD Anderson Cancer Center, Unit 1460, 1515 Holcombe Blvd, Houston, TX, 77030, 713-792-3629 (o), 713-792-5381 (fax), sshelburne@mdanderson.org

Bovine Leukemia Virus DNA in Human Breast Tissue

Gertrude Case Buehring, Hua Min Shen, Hanne M. Jensen, K. Yeon Choi,[1] Dejun Sun, and Gerard Nuovo

Bovine leukemia virus (BLV), a deltaretrovirus, causes B-cell leukemia/lymphoma in cattle and is prevalent in herds globally. A previous finding of antibodies against BLV in humans led us to examine the possibility of human infection with BLV. We focused on breast tissue because, in cattle, BLV DNA and protein have been found to be more abundant in mammary epithelium than in lymphocytes. In human breast tissue specimens, we identified BLV DNA by using nested liquid-phase PCR and DNA sequencing. Variations from the bovine reference sequence were infrequent and limited to base substitutions. In situ PCR and immunohistochemical testing localized BLV to the secretory epithelium of the breast. Our finding of BLV in human tissues indicates a risk for the acquisition and proliferation of this virus in humans. Further research is needed to determine whether BLV may play a direct role in human disease.

Bovine leukosis (B-cell leukemia/lymphoma), first described in 1871 in Lithuania, was believed to be an infectious disease because it spread through herds of cattle. In 1969, a virus isolated from cultured lymphocytes of cattle in an afflicted herd was identified as the agent of bovine leukosis (*1*). Since then, bovine leukemia virus (BLV) has been extensively investigated. It is a deltaretrovirus, closely related to human T-cell leukemia virus (HTLV) 1 (*2*), and has typical retroviral genome regions: *LTR* (long terminal repeat, promoter region); *gag* (group-specific antigen, capsid region); *pol* (polymerase, reverse transcription region, which synthesizes a DNA copy of the BLV RNA genome); and *env* (envelope). However, unlike other oncogenic retroviruses, deltaretroviruses have an additional region, *tax* (trans-activating region of the X gene), which has regulatory functions and is oncogenic to host cells. *tax* causes malignant transformation not through integration and insertional mutagenesis, as many retroviruses do, but by inhibition of DNA repair (base excision pathway) and trans-activating disruption of cellular growth control mechanisms (*2*).

BLV-infected cattle herds are found worldwide. In the United States, ≈38% of beef herds, 84% of all dairy herds, and 100% of large-scale dairy operation herds are infected (*3*,*4*). On average, clinical leukosis develops in <5% of these cattle, which are excluded from the market as a result (*1*), but BLV-infected lymphocytes are also found in the blood and milk of subclinically infected cows (*2*). Concerns that this virus might infect humans through exposure to food products from subclinically infected animals prompted 10 studies that used what were then (1975–1979) state-of-the-art immunologic methods to test serum samples from a collective total of 1,761 humans, including cancer patients, farm workers, and veterinarians (*5*). In these studies no antibodies against BLV were detected, prompting Burridge to conclude in his review article, "There is no epidemiological or serological evidence from human studies to indicate that BLV can infect man" (*5*).

The advent of immunoblotting, ≈100 times more sensitive than techniques of the 1970s (*6*), enabled the detection of antibodies reactive with recombinant purified BLV p24 capsid protein in serum samples from 39% of 257 self-selected human volunteers (*7*). This study could not determine whether the antibodies were a response to infection or merely to heat-inactivated BLV consumed in food products. However, injection of sheep with raw milk from BLV-positive cows stimulated antibody production,

Author affiliations: University of California, Berkeley, California, USA (G.C. Buehring, H.M. Shen, K.Y. Choi, D. Sun); University of California Davis Medical Center, Sacramento, California, USA (H.M. Jensen); and Ohio State University Comprehensive Cancer Center, Columbus, Ohio, USA (G. Nuovo)

DOI: http://dx.doi.org/10.3201/eid2005.131298

[1]Current affiliation: Texas A&M Health Science Center College of Medicine, College Station, Texas, USA.

whereas injection with pasteurized control milk did not (*8,9*). The finding of antibodies to BLV in humans prompted us to investigate human tissues for evidence of infection with BLV by using liquid-phase PCR (L-PCR), sequencing, in situ PCR, and immunohistochemical (IHC) testing. We focused on breast tissue because, in cattle, BLV DNA and p24 were detected in mammary tissue, whereas only BLV DNA was detected in lymphocytes (*10*).

Materials and Methods

Cells and DNA used to test cross-reactivity of BLV primers and anti-BLV p24 monoclonal antibody (mAb) with other viruses are listed with their sources in Tables 1 and 2. Except for fetal lamb kidney (FLK) cells, cell lines were stored in liquid nitrogen until use. Before experiments were performed, the species of origin of all cell lines was authenticated by using the 2-pronged method (cytochrome oxidase housekeeping gene primers) (*11*).

Coded human samples were acquired from the Cooperative Human Tissue Network, a National Cancer Institute–supported tissue bank. Specimens were selected, without regard to patient age, race, or diagnosis, from archived breast tissues acquired from female patients who underwent breast surgery during 2000–2005 at participating hospitals in 4 catchments areas: Birmingham, Alabama; Pennsylvania; Ohio; and Oakland, California. The Institutional Review Board of the University of California, Berkeley (Berkeley, CA, USA) approved human subject use. Bovine control tissue came from the University of Wisconsin, Madison (Madison, WI, USA), as described (*10*).

PCR primers (Table 3) were examined for BLV specificity by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for similar sequences in the nucleotide collection database, which includes exogenous viruses and human endogenous retroviruses such as HERV-K. The search was optimized for highly similar sequences.

For DNA extraction and quality control, cell lines were rinsed with Dulbecco phosphate-buffered saline (DPBS) and pelleted (500 × *g* for 3–5 min); DNA was then extracted by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's cell protocol. DNA from human tissue specimens was extracted from frozen or deparaffinized formalin-fixed paraffin-embedded (FFPE) sections (5 μm thick) by using the QIAamp DNA Mini Kit according to the manufacturer's tissue protocol. Overnight proteinase K digestion was extended 3–6 h to result in complete digestion, free of visible tissue particles. Extracted DNA quality

Table 1. Lack of cross-reactivity of primers from 5 BLV genome regions with representatives of mammalian and avian retrovirus subfamilies and human exogenous and endogenous viruses previously identified in human breast tissue*

Virus	Subfamily	Cell line harboring virus (source†)	Nested liquid-phase PCR for BLV genome regions				
			LTR	*gag*	*pol*	*env*	*tax*
RSV	Alpharetrovirus	XC, rat cell line, transformed with RSV (CCL/NBRL)	–	–	–	–	–
MSV	Alpharetrovirus	F81, cat cell line, MSV infected (CCL/NBRL)	–	–	–	–	–
MMTV	Betaretrovirus	GR, mouse mammary tumor cell line (G. Firestone)	–	–	–	–	–
MPMV	Betaretrovirus	CMMT, rhesus monkey cell line (CCL/NBRL)	–	–	–	–	–
MuLV	Gammaretrovirus	JLSV5, mouse cell line (CCL/NBRL 14)	–	–	–	–	–
FeLV	Gammaretrovirus	FeLV 3281, cat cell line (CCL/NBRL)	–	–	–	–	–
BLV	Deltaretrovirus	FLK cell line (K. Radke)	+	+	+	+	+
		Bat_2Clone_6 cell line, BLV infected (K. Radke)	+	+	+	+	+
None		Tb_1Lu, parental line of Bat_2Clone_6 before it was infected with BLV (K. Radke)	–	–	–	–	–
STLV	Deltaretrovirus	KIA, baboon cell line (ARRRP)	–	–	–	–	–
HTLV-1	Deltaretrovirus	MT2, human lymphocyte cell line (C. Hanson)	–	–	–	–	–
HTLV-2	Deltaretrovirus	Clone 19, human lymphocyte cell line (C. Hanson)	–	–	–	–	–
HIV-1	Lentivirus	H9, human cell line, HIV-1 infected (C. Hanson)	–	–	–	–	–
HIV-2	Lentivirus	H9, human cell line, HIV-2 infected (C. Hanson)	–	–	–	–	–
HPV-16	Papillomavirus	Caski, human uterine cervix cell line (ATCC)	–	–	–	–	–
HPV-18	Papillomavirus	HeLa, human uterine cervix cell line (CCL/NBRL)	–	–	–	–	–
EBV	Gamma-1 herpesvirus	Raji, human B-cell line (ATCC)	–	–	–	–	–
HERV-K	HERV, Class II	MCF-7, human breast cell line (ATCC)	–	–	–	–	–
		Purified, cloned HERV-K DNA (F. Wang-Johanning)	–	–	–	–	–

*In addition to assurances from the sources of the above biologicals, presence of the viruses in the respective cell lines was supported by rescue/syncytia formation when cell lines with replication defective viruses (RSV and MSV) were co-infected with replication competent retroviruses; positive reaction with primers specific for the virus (BLV, HTLV-1, -2, EBV, HPV-16); reaction with antibodies to the respective virus (MMTV, MMPV, MuLV); or receipt as formalin-fixed specimen so virus could not have been altered or lost (HIV-1, -2). BLV, bovine leukemia virus; *LTR*, long terminal repeat (promoter region); *gag*, group-specific antigen (capsid region); *pol*, polymerase (reverse transcription); *env*, envelope; *tax*, trans-activating region of the X gene; RSV, Rous sarcoma virus; MSV, murine sarcoma virus; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; MuLV, murine leukemia virus; FeLV, feline leukemia virus; FLK, fetal lamb kidney; STLV, simian T-cell leukemia virus; HTLV, human T-cell leukemia virus; HPV, human papillomavirus; EBV, Epstein-Barr virus; HERV, human endogenous retrovirus.
†Individually named sources listed in Acknowledgments. CCL/NBRL, former Cell Culture Laboratory of the Naval Bioscience Research Laboratory, Oakland, CA, USA; ARRRP, AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (www.aidsreagent.org); ATCC, American Type Culture Collection.

Table 2. Lack of cross-reactivity of anti-BLV p24 (capsid region) mAb with common chronic viruses in human tissues*

Virus	Cells or tissue producing virus (source†)	Type of antibody to virus (source†)	Reaction of cells or tissue with	
			Ab to virus	mAb to BLV p24
BLV	FLK (positive control) (K. Radke)	mAb to BLV p24 (ARRRP no. 12145)	+	+
	Bat_2Cl_6 (positive control) (K. Radke)		+	+
None	Tb_1Lu (negative control) (K. Radke)		–	–
MMTV	GR (G. Firestone)	pAb to MMTV (NCI-BCP)	+	–
HBV	Infected human liver sections (Dako)	pAb to HBV (Dako)	+	–
HTLV-1	MT-2 (C. Hanson)	pAb to HTLV-1 (ARRRP)	+	–
HTLV-2	Clone-19 (C. Hanson)	pAb to HTLV-2 (ARRRP)	+	–
HIV-1, -2	H9 HIV-1, -2 (C. Hanson)	pAb to HIV-1, -2 (ARRRP)	+	–
HPV	HPV-infected keratinocyte cultures (C. Meyers)	pAb to PV (Dako)	+	–
EBV	B95–8 (CCL/NBRL)	pAb to EBV (Dako)	+	–
CMV	Infected human lung sections (Dako)	pAb to CMV (Chemicon/Millipore)	+	–
HHV 1, 2	Infected human cells (Syva)	pAb to HHV 1, 2 (Chemicon/Millipore)	+	–

*BLV, bovine leukemia virus; mAb, monoclonal antibody; Ab, antibody; FLK, fetal lamb kidney; MMTV, mouse mammary tumor virus; pAb, polyclonal antibody; HBV, hepatitis B virus; HTLV, human T cell leukemia virus; HPV, human papillomavirus; PV, papillomavirus; EBV, Epstein-Barr virus; CMV, cytomegalovirus; HHV, human herpes virus.
†Individually named sources listed in Acknowledgments. ARRRP, AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (www.aidsreagent.org); NCI-BCP, National Cancer Institute, Breast Cancer Program; Dako, Glostrup, Denmark; CCL/NBRL, former Cell Culture Laboratory of the Naval Bioscience Research Laboratory, Oakland, CA, USA; Chemicon/Millipore, Billerica, MA; Syva, Palo Alto, CA, USA.

was confirmed by amplification of a housekeeping gene sequence: human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for human, rhesus monkey, baboon, and bat material; murine GAPDH for mouse and rat cell lines; and bovine GAPDH for bovine, ovine, and feline cell lines (Table 4). Molecular contamination of extracted human DNA by BLV control DNA was monitored by using sheep-specific primers for the FLK cell line and plasmid vector primers for the C72/*tax*Neo line (bovine mammary epithelial cells stably transfected with BLV *tax* gene) (*12*), the only 2 BLV-containing cell lines previously grown in the laboratory in which testing was conducted.

Table 3. BLV primers and cycling conditions used for L-PCR, IS-PCR, and preparation of DNA for sequencing for detection of BLV in human breast tissue samples*

BLV gene	Primer pair sequences, 5′ → 3′†	Location in bp‡	Nested PCR role	Product length, bp	L-PCR/IS-PCR§	
					Annealing temperature, °C	Extension time, s
LTR	F: TAGGAGCCGCCACCGC	23–38	Outer	329	57/53	22/120
	R: GCGGTGGTCTCAGCCGA	352–336				
	F:AAACTGCAGCGTAAACCAGACAGAGACG	41–59	Inner	290	58/57	20/120
	R: CACCCTCCAAACCGTGCTTG	331–312				
gag (p24)	F: AACACTACGACTTGCAATCC	1068–1087	Outer	385	54/53	28/120
	R: GGTTCCTTAGGACTCCGTCG	1453–1434				
	F: ACCCTACTCCGGCTGACCTA	1097–1116	Inner	272	56/56	24/120
	R: CTTGGACGATGGTGGACCAA	1369–1350				
pol	F: TAGCCTACGTACATCTAACC	3238–3257	Outer	232	52/53	22/120
	R: AATCCAATTGTCTAGAGAGG	3470–3451				
	F: GGTCCACCCTGGTACTCTTC	3265–3284	Inner	157	57/56	18/120
	R: TATGGGCTTGGCATACGAGC	3422–3403				
env	F: TGATTGCGAGCCCCGATG	5144–5160	Outer	264	55/53	24/120
	R: TCTGACAGAGGGAACCCAGT	5408–5389				
	F: TGATTGCGAGCCCCGATG	5144–5160	Inner	230	55/56	22/120
	R: GGAAAGTCGGGTTGAGGG	5374–5357				
tax	F: CTTCGGGATCCATTACCTGA	7197–7216	Outer	373	55/55	26/120
	R: GCTCGAAGGGGGAAAGTGAA	7570–7551				
	F: ATGTCACCATCGATGCCTGG	7310–7329	Inner 1	113	55/53	15/120
	R: CATCGGCGGTCCAGTTGATA	7423–7404				
	F: GGCCCCACTCTCTACATGC	7265–7283	Inner 2	206	56	22
	R: AGACATGCAGTCGAGGGAAC	7471–7452	(sequencing)			

*BLV, bovine leukemia virus; L-PCR, nested liquid-phase PCR; IS-PCR, nested in situ PCR; *LTR*, long terminal repeat (promoter region); F, forward primer; R, reverse primer; *gag*, group-specific antigen (capsid region); *pol*, polymerase (reverse transcription); *env*, envelope; *tax*, trans-activating region of the X gene.
†Reverse sequences are reversed and complementary to the proviral reference sequence. Primers were synthesized by Operon Biotechnologies, Huntsville, AL, USA.
‡Location according to reference sequence, GenBank accession no. EF600696.
§For both rounds of nested liquid-phase PCR, cycling conditions were as follows: 1 cycle at 95°C for 2 min; 30 cycles at 95°C for 30 s, XX°C for 30 s, 72°C for XX s; and 1 cycle at 72°C for 5 min, where XX represents annealing temperature and extension times, respectively, provided for each primer pair. For both rounds of nested in situ PCR, conditions were as follows: 1 cycle at 92°C for 10 min, 1 cycle at 91°C for 2 min, XX°C for 1.5 min; then 30 cycles of 91°C for 30 s, XX°C for 1.5 min, 72°C for 2 min; and 1 cycle of 72°C for 10 min, where XX represents IS-PCR annealing temperatures indicated for each primer pair.

Table 4. Primers and reaction conditions for GAPDH gene amplifications used to verify quality of BLV DNA extracted from human breast tissue samples, human cell lines, and animal cell lines*

Gene	Primer pair sequences, 5′ → 3′†	Location in bp‡	Product length, bp	Annealing temperature, °C§	Extension time, s§
Human GAPDH	F: GAGTCAACGGATTTGGTCGT	194–213	237	50	22
	R: TTGATTTTGGAGGGATCTCG	431–412			
Mouse GAPDH	F: AGCTTGTCATCAACGGGAAG	246–265	796	58	60
	R: ATGTAGGCCATGAGGTCCAC	1041–1022			
Bovine GAPDH	F: CCTTCATTGACCTTCACTACATGGTCTA	172–199	857	59	60
	R: GCTGTAGCCAAATTCATTGTCGTACCA	1028–1002			

*GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BLV, bovine leukemia virus; F, forward primer; R, reverse primer.
†Reverse sequences are reversed and complementary to the published genomic sequences. Primers were synthesized by Operon Biotechnologies, Huntsville, AL, USA.
‡Sequence bp numbering according to GenBank no. NM 002046.4 (human), XM 001476707.3 (mouse), and NM 001034034.2 (bovine).
§Cycling conditions were 1 cycle at 95°C for 2 min; 35 cycles at 95°C for 30 s; XX°C for 30 s, 72°C for XX s; and 1 cycle at 72°C for 5 min, where XX represents annealing temperature and extension times, respectively, for primer pairs.

For nested L-PCR, extracted DNA (0.85 μg) was added to 50 μL of PCR mix (2.0 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 0.025 U/μL Taq polymerase [all from Promega, Madison, WI, USA], and 0.2 μmol/L outer primers for each BLV gene [Table 3]) in Hot Start Micro 50 tubes (MolecularBio Products, San Diego, CA, USA). For the second (nested) round of amplification, 2 μL of the first-round PCR product was added to a new tube of the same reaction mix with inner primers for the corresponding genome region (Table 3). Cycling conditions are given in Table 3. Reaction mix and conditions, including $MgCl_2$ concentrations, were optimized by using the BLV-positive FLK fibroblasts and BLV-negative bat lung fibroblasts (Tb_1Lu). Sensitivity of the nested L-PCR was determined by using the housekeeping gene GAPDH, which occurs in humans as 1 copy/cell (*13*). DNA was extracted from 1 million cells of the human cell line MCF7 (Table 1) and serially diluted to an end point equivalent of 1 cell. Nested L-PCR performed on each dilution established the sensitivity at ≤10 copies.

L-PCR, which requires initial tissue digestion to extract DNA, is prone to molecular contamination. We reduced this probability by separating PCR procedure steps into 3 locations: 1) preparation of PCR mixes in a dedicated, locked, DNA-free room with entrance and ventilation system separate from the main laboratory and with dedicated laminar flow hood, small equipment, plasticware, and reagents; 2) addition of template DNA in a different room in a small hood dedicated to specimen handling, with DNA easily decontaminated by UV light and DNA decontamination solution (RNase AWAY; Molecular BioProducts, San Diego, CA, USA) applied to equipment and surfaces before and after handling each specimen; and 3) processing and addition of positive control samples last, and only in a fume hood (velocity = 127 fpm) vented to atmospheric air, thus preventing contaminating aerosols from entering the general work area.

For sequencing, BLV amplicons obtained by nested L-PCR were separated by electrophoreses in a 1% agarose gel at 100 V, excised from the gel, and cleaned by using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). The requested sample of 100 ng of DNA in 12 μL water and 1 μL of the 5 μmol/L sequencing primer stock solution of the inner primer pair for each genome region (Table 3) was submitted to the University of California, Berkeley, DNA Sequencing Facility for direct sequencing. For *tax*, inner primer 2 was used to obtain a longer product. DNA for sequencing was obtained from 2–5 amplifications and was sequenced at least once in forward (5′) and reverse (3′) directions. Sequences were checked against corresponding electropherograms. Variations from the reference sequence (GenBank accession no. EF600696) were considered valid only if they matched in both forward and reverse directions. Targeted sequences were relatively short because formalin causes DNA breaks, making it difficult to obtain sequences of >130 nt (*14*).

Nested in situ PCR (IS-PCR), adapted from Nuovo (*15*), was used to identify which cell types within tissues were BLV positive. Thick suspensions of detached, rinsed control cells were smeared on enhanced adherence glass microscope slides, air dried, and fixed for 16–18 h with 10% buffered neutral formalin. To enhance entry of PCR mix into cells, samples were made permeable by digestion with 2 mg/mL pepsin in 0.1 N HCl (40–80 min for tissue sections; 20 min for control cell smears), pepsin inactivation solution (100 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.4) applied for 1 min, followed by a DPBS rinse and 5 min in absolute ethanol. Samples, run in duplicate, were surrounded with a 15 × 15 mm frame seal chamber (Bio-Rad, Hercules, CA, USA); 60 μL of PCR mix were then placed into the chamber, and the plastic cover was sealed over the frame. The PCR mixture was 4.0 mmol/L $MgCl_2$, 0.4 mmol/L dNTPs, 1 μmol/L primers (Operon Biotechnologies, Huntsville, AL, USA), 0.06% bovine serum albumin, 8 μmol/L digoxigenin-11-dUTP (dig) (Hoffman-La Roche, Basel, Switzerland), and 0.053 U/μL Amplitaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), a Taq polymerase activated only at ≥92°C, designed to reduce false positives from nonspecific DNA repair by Taq at cooler temperatures (*15*). Primers were the same as those used for L-PCR (Table 3). Slides were placed into an

IS-PCR machine (Hybaid Thermo OmniSlide; Cambridge Biosystems, Cambridge, UK) for amplification. After each round, covers and chambers were removed, and slides were rinsed in DPBS. After the second round, endogenous peroxidase was quenched 30 min in 3% H_2O_2 in methanol. Label incorporated into PCR products was detected by anti-dig antibodies in an avidin-biotin-immunoperoxidase reaction (Hoffman-La Roche). The chromagen was diaminobenzidine. Outcome measurement was a semiquantitative judgment of color density of cells: 1+, light tan; 2+, medium tan; 3+, dark brown; 4+, almost black. Ratings of ≥2+ were considered positive. No donor information or results of L-PCR were available at the time of slide evaluation.

Initially, the BLV-positive control cell line (FLK) and the BLV-negative cell line (Tb_1Lu) were used to optimize the reaction and ensure no false-positive reaction in the negative cell line. Control testing was run simultaneously with each batch of human tissue assays: 1) positive control, a smear of BLV-positive cells (FLK cell line) reacted with complete PCR mix; 2) negative controls, a smear of FLK cells and an adjacent serial section of each specimen reacted with PCR mix minus primers, to rule out false-positive reactions unique to each tissue resulting from unquenched endogenous peroxidase, nonspecific reaction of the sheep antibodies used in the final immunoperoxidase detection, or nonspecific DNA repair by Taq polymerase; 3) permeabilization control for entry of PCR mix into cells, with an adjacent serial section of each tissue reacted with PCR mix different from that for IS-PCR by omission of primers, 4.5 mmol/L concentration for $MgCl_2$, and use of a different Taq polymerase (Promega) that reacts nonspecifically at cooler temperatures (4°C–50°C) to repair DNA. Specimens were scored positive only if mammary epithelial cells were positive and the background control slide (adjacent section) without primers was negative for the corresponding area of mammary epithelial cells. Specimens were scored negative only if the sample and its background control section were negative and the permeabilized control exhibited dig incorporation into cell nuclei resulting from Taq polymerase DNA repair, confirming entry of PCR mix into cells (*15*).

IHC testing for BLV p24 was performed by using formalin-fixed cell smears and deparaffinized FFPE tissue sections (5 μm) on superadherent microscope slides. Samples were quenched of endogenous peroxidase for 30 min in 3% H_2O_2 in methanol and rinsed in DPBS; antigens were then unmasked by incubation in citrate buffer (0.1 mmol/L sodium citrate, 0.04 mmol/L citric acid, pH 6.0) for 25 min at 95°C in individual plastic containers to prevent cross-transfer of tissue material. Unmasking was followed by a DPBS rinse, and an avidin-biotin-immunoperoxidase procedure (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) was performed according to the manufacturer's instructions. Blocking serum was 1.5% fetal horse serum in DPBS. Primary antibody in blocking serum (1:10) was a hybridoma-produced mouse mAb against BLV p24 (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases; www.aidsreagent.org) (*16*); specificity was validated by immunoblot reactivity with purified recombinant p24 (*7*). Secondary antibody was 1.5% horse anti-mouse IgG (Vector) in blocking serum. The chromagen was diaminobenzidine. Negative controls were the BLV-negative control cell line Tb1Lu and adjacent human tissue sections reacted with fresh hybridoma medium (diluted 1:10 in blocking buffer) substituted for primary antibody. The positive control was FLK, which is known to replicate BLV. Outcome measurement was a semiquantitative judgment of color density, as described for IS-PCR.

Results

Human samples were selected from 219 FFPE breast tissue samples; 97 (44%) samples had positive results for BLV by nested IS-PCR that used primers from the most conserved BLV genome region, *tax*. Five *tax*-positive samples and 1 *tax*-negative sample (as a control) were chosen for in-depth molecular analysis on the basis of BLV status and sample size large enough and with enough mammary epithelial cells to yield sufficient material for multiple assays and extensive quality control tests. BLV was detected in DNA extracted from human tissues by using nested L-PCR. All 5 *tax*-positive samples chosen were positive for the *LTR* region but showed varying results for other BLV genome regions (Table 5; Figure 1). Sequences of all samples positive for BLV had high identity (E value ≤1.2) only to BLV nucleotide sequences deposited in GenBank, which suggests that these isolates did not represent some other

Table 5. PCR results for detection of BLV in breast tissue samples from 6 women*

			BLV genome regions					
			L-PCR					IS-PCR
Sample code	Sample pathology	Patient age, y	*LTR*	*gag*	*pol*	*env*	*tax*	*tax*
143	Malignant	63	–	–	–	–	–	–
0253	Malignant	47	+	–	–	–	+	+
010	Malignant	48	+	–	–	–	+	+
236	Nonmalignant	54	+	+	–	+	+	+
23803	Nonmalignant	50	+	+	–	+	+	+
20874	Nonmalignant	53	+	+	+	+	+	+

*BLV, bovine leukemia virus; L-PCR, nested liquid-phase PCR; IS-PCR, nested in situ PCR; *LTR*, long terminal repeat (promoter region); *gag*, group-specific antigen (capsid region); *pol*, polymerase (reverse transcription); *env*, envelope; *tax*, transactivating region of the X gene.

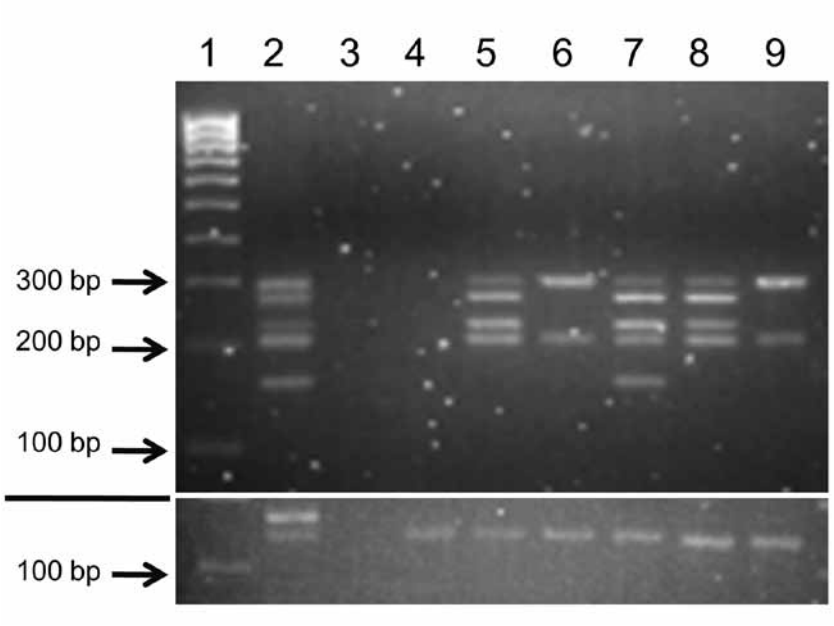


Figure 1. Amplification of bovine leukemia virus (BLV) genome regions in human breast tissue specimens. Nested liquid-phase PCR, using primers from 5 BLV genome regions, was used to amplify products from DNA extracted from breast tissues of 6 human donors. PCR products for each tissue were loaded into 1 well and separated by agarose gel (3.5%) electrophoresis on the basis of size differences: long terminal repeat, 290 bp; group-specific antigen, 272 bp; envelope, 230 bp; trans-activating gene of the X region, 206 bp; polymerase, 157 bp. The section below the white line shows the glyceraldehyde 3-phosphate dehydrogenase amplification of each sample as an indicator of DNA quality. Lane 1, molecular weight marker (HyperLadder IV; Bioline, Taunton, MA, USA); lane 2, fetal lamb kidney cell line, positive control; lane 3, no-template-DNA negative control (water substituted for DNA template); lane 4, human sample 143; lane 5, human sample 236; lane 6, human sample 010; lane 7, human sample 20874; lane 8, human sample 23803; lane 9, human sample 0253.

entity. Variations from the BLV reference sequence were infrequent, and all involved base substitutions (Figure 2).

Nested IS-PCR was used to identify the cell type in which the PCR product was localized. Figure 3 shows IS-PCR results obtained by using *tax* primers for the BLV-negative human sample (no. 143), 1 of the BLV-positive samples (no. 010), the positive and negative cell line controls, and a BLV *tax*-positive bovine mammary gland sample. The site of the amplified BLV DNA was the secretory mammary epithelium, identified by an anatomic pathologist (H.M.J.). Table 5 summarizes the results for L-PCR and IS-PCR (*tax*) testing of the 6 tissue samples that were studied in depth.

Validation of the IS-PCR results for a subset of 7 samples (3 *tax* negative and 4 *tax* positive) was performed by an independent laboratory by using control cell smears and coded FFPE sections sent from our laboratory with no information about the human patients, tissue pathology, or our results. The detection method was PCR in situ hybridization, in which the PCR occurs in situ but with no label incorporated during amplification (*15*). Labeled probes specific for the BLV *tax* region were applied after amplification. The independent laboratory confirmed with 100% concordance the results we obtained by using nested IS-PCR and L-PCR for the *tax* genome region (Table 6).

Of the 215 human breast tissue samples tested by IHC, 12 (6%) had positive results for BLV p24. The reaction was confined to secretory mammary epithelium, and distribution of both BLV DNA and p24 in mammary epithelial cells was duct and lobule specific; that is, virtually all cells in the duct or lobule would be BLV positive but neighboring ducts or lobules could be completely negative. A BLV p24–positive breast tissue sample is shown in Figure 3, panel F.

GenBank searches indicated complete identity (E value ≤1.1) of PCR primers (Table 3) only with BLV sequences. L-PCR further substantiated primer specificity (Figure 4; Table 1) by demonstrating no cross-reactivity (amplicon generation) by using template DNA from cell lines harboring representatives of all mammalian and avian oncogenic retroviral subfamilies and human lentiviruses; viruses previously reported in human breast tissues (Epstein-Barr virus, human papillomavirus, mouse mammary tumor virus–like sequences) (*17*); and human endogenous retrovirus HERV-K (Table 1). Extracted DNA met quality control standards of exhibiting a housekeeping gene (GAPDH; Figure 4). mAb specificity was supported by lack of cross-reactivity with cell lines or by tissues replicating viruses previously identified in human breast tissues (Epstein-Barr virus, human papillomavirus, and mouse mammary tumor virus–like sequences) (*17*) or that cause chronic human infections (hepatitis B virus, human T-cell leukemia viruses 1 and 2, HIV 1 and 2, human herpesviruses 1 and 2, and cytomegalovirus) (Table 2).

Several methods were used to test for cell and molecular contamination. The no-template-DNA control run with each assay ruled out contamination in commercial DNA isolation columns and PCR reagents; in addition, contamination of human DNA samples by FLK DNA was ruled out by testing human DNA with sheep-specific

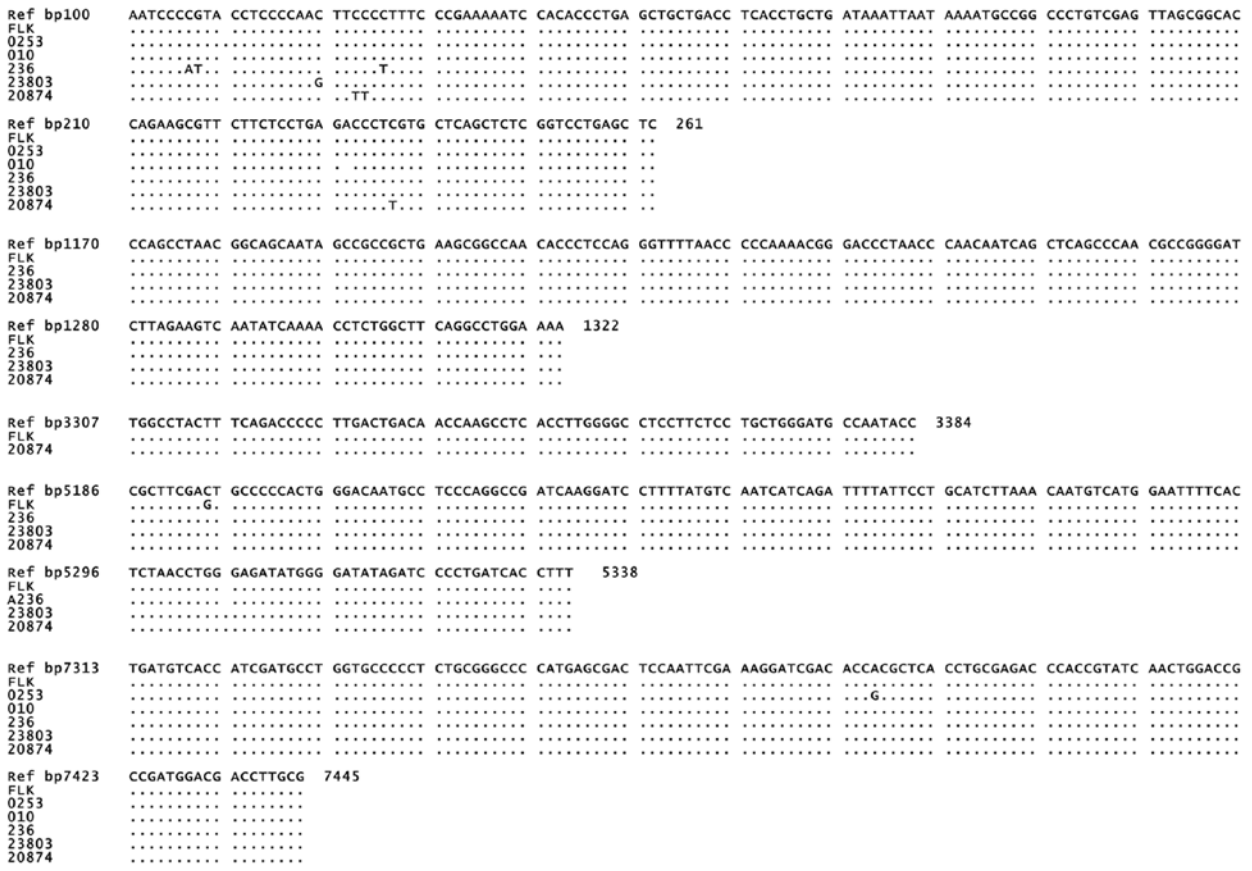


Figure 2. Partial sequences of 5 genome regions of bovine leukemia virus (BLV) DNA isolated from human breast tissue samples: A) long terminal repeat; B) group-specific antigen; C) polymerase; D) envelope; E) trans-activating gene of the X region. Reference (Ref) sequences (GenBank accession no. EF600696) are shown; dots indicate where samples have no difference from the reference sequence. Fetal lamb kidney (FLK) is shown as positive control cell line. Human samples are listed by sample number. Sequences displayed are shorter than the amplicon each primer set amplified because only overlapping regions of both forward and reverse sequencing are shown. Missing sequences indicate that the sample did not test positive for those genome regions.

primers (*11*), and contamination of FLK with other cell lines, including human, was ruled out by using species-specific primers (*11*). Sequences from the human isolates also provided strong evidence against contamination; none were exact matches with each other or with any Genbank BLV sequence, which includes the FLK-positive control cell line and bovine specimens previously sequenced in our laboratory. Furthermore, the FLK stock cell line has a signature base substitution (bp 5194; GenBank accession no. EF600696) in the *env* region; this substitution is unique among sequences deposited in GenBank and was not detected in the human BLV isolates we investigated (Figure 2). The positive results obtained by using in situ methods (IHC and IS-PCR) demonstrating the signal within individual formalin-fixed mammary epithelial cells are further evidence that the positive reactions do not represent contamination of PCR mix. Previous reports have documented that contaminating DNA cannot cause a false-positive reaction with PCR in situ hybridization in FFPE tissues (*15*). Negative controls (adjacent tissue sections without BLV-specific primers or antibody) run with each in situ assay were all negative, which suggests that positive signals were not false positives resulting from nonspecific reactions.

Discussion

During the 4 decades since BLV was identified in cattle, there has been considerable interest in determining whether humans could become infected with BLV, especially because cattle are a major food source. As in early serologic studies, previous cellular and molecular studies reported no evidence of human infection with BLV. An explanation for some of the negative findings (*18–22*) could be that sufficiently sensitive reagents and techniques such as PCR and sequencing were not available when the studies were conducted. Most previous studies also focused on leukocytes, the cell type involved in bovine leukemia/lymphoma (*18–27*). We focused instead on mammary

epithelium, in which we had detected BLV DNA and protein in cattle (*10*). In situ techniques (i.e., IS-PCR, PCR in situ hybridization, and IHC) enabled confirmation that BLV was localized within mammary epithelial cells.

Evidence for BLV DNA and protein in humans is not surprising. Many viruses, including those that are oncogenic, are known to cross species naturally, and most microbial species pathogenic in humans are speculated to have had a animal origin at some point in human evolution. Once in the human population, most zoonotic viruses can be transmitted among humans, a process that poses the most serious threat to human health (*28*). BLV is known to cross

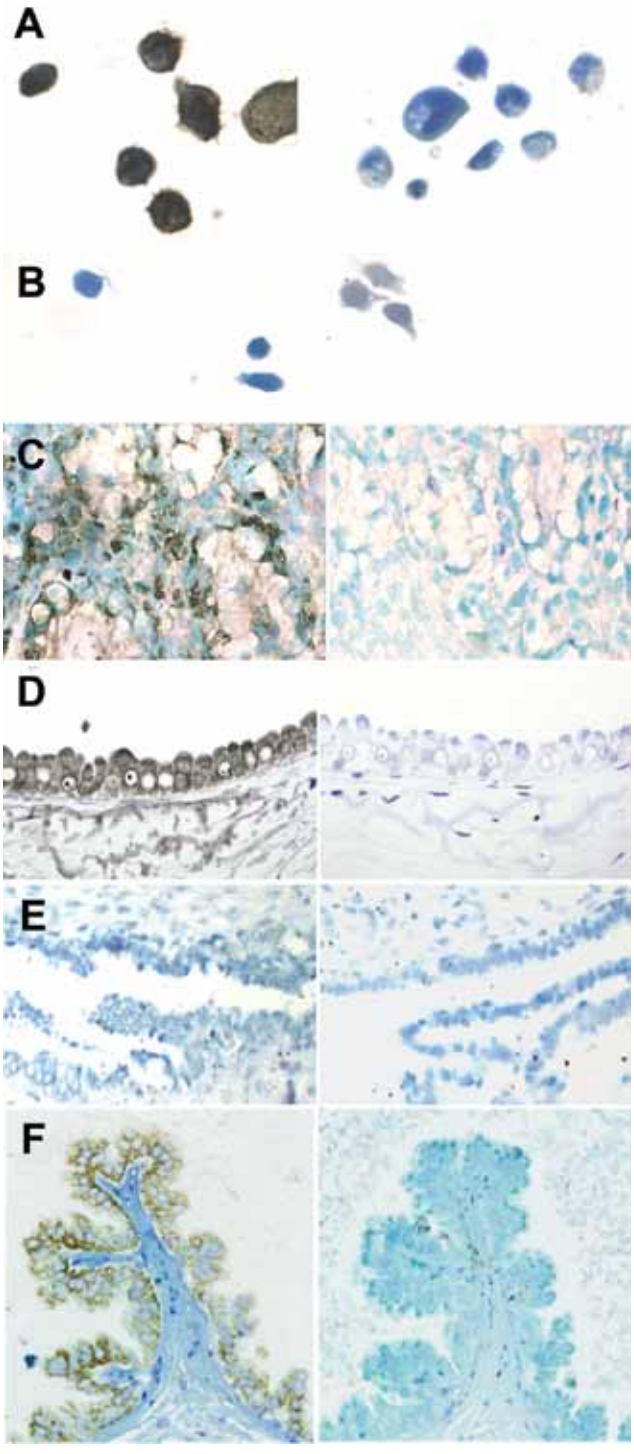


Figure 3. Localization of bovine leukemia virus (BLV) in human breast tissue and bovine mammary epithelium samples detected by in situ PCR for the BLV *tax* region and immunohistochemical testing for p24 capsid protein. A) BLV-positive fetal lamb kidney (FLK) cell line. Brown at left indicates positive diaminobenzidine endpoint immunoperoxidase reaction to detect digoxygenin incorporated into PCR product within FLK cells. FLK cells reacted with PCR mix without primers (right) to check for false-positive background show no reaction. Original magnification ×400. B) BLV-negative cell line Tb_1Lu with (left) and without (right) primers. No reaction occurred with either condition because the cell line has no BLV to amplify and shows no nonspecific background. Original magnification ×400. C) BLV-positive lactating bovine mammary gland tissue with (left) and without (right) *tax* primers in the PCR mix. Dark brown at left indicates positive cells, some surrounding lumens filled with milk. Lack of reactive cells in sample at right without primers indicates reaction was not a false positive due to nonspecific factors inherent in the tissue. Original magnification ×100. D) BLV-positive human tissue sample 010 reacted with *tax* primers. Dark brown at left indicates epithelial cells facing the lumen of a large cyst. Lack of reactive cells in sample at right without primers indicates reaction was not a false positive. Original magnification ×100. E) BLV-negative human tissue sample 143 exposed to PCR mix with (left) and without (right) primers showing no reaction with either condition in the epithelium of the long duct. Original magnification ×40. F) BLV-positive human tissue reacted with monoclonal antibody to BLV p24 (left) in an avidin-biotin-immunoperoxidase assay. Dark color indicates end-point reaction in cytoplasm of epithelium projecting into the cyst lumen on a stalk of collagenous stroma. Note lack of reaction in sample at right with hybridoma medium substituted for primary antibody. Original magnification ×40. All cells and tissues were counterstained with Diff-Quik Solution II (Dade Behring, Newark, DE, USA).

Table 6. Validation of PCR results for representative breast tissues tested at the University of California Berkeley, conducted at an independent laboratory*

Sample code	Sample pathology	IS-PCR result	PCR in situ hybridization result†
B702	Nonmalignant	–	–
143‡	Malignant	–	–
B984	Malignant	–	–
236‡	Nonmalignant	+	+
B975	Nonmalignant	+	+
154	Malignant	+	+
253‡	Malignant	+	+

*IS-PCR, nested in situ PCR.
†Ohio State University Comprehensive Cancer Center, Columbus, OH, USA.
‡Samples were among those used for in-depth study reported in this article (Table 5).

species readily; the virus infects capybara, zebus, and water buffaloes naturally and sheep, goats, pigs, rabbits, rats, and chickens experimentally (*1*). Human cells (fibroblasts) are susceptible to infection with BLV in vitro (*29*).

The lack of *gag, pol*, and *env* sequences in some of the BLV-positive panel samples and the presence of *LTR* and *tax* sequences in all of them is consistent with results reported for the closely related HTLV-1 (*30*). Deletions in *gag, pol,* and *env* were observed in HTLV-1 isolates from 25.7%–56% of adult T-cell leukemia patients, and frequency increased with clinical progression of leukemia (*30*). Such deletions are postulated to be advantageous to the virus by enabling escape from immune surveillance. In contrast, the *LTR* and *tax* regions of HTLV-1 are highly conserved. Analogous results were observed in specimens from cattle infected with BLV; deletions involving parts of *gag* and *env* and all of *pol* were frequent (*2*).

Overall, the human BLV isolates differed from the reference sequence by only a few base substitutions, a finding that fits well with the biology of deltaretroviruses, which have infrequent interhost transmission and remain largely latent within the host, probably as a strategy to escape the host's immune response (*31*). Infectivity of deltaretroviruses occurs primarily by cell–cell contact, not by extracellular virions (*32*), and BLV virions have not been found in the peripheral blood of infected cattle (*2*). In our study, evidence of the capsid protein p24 was infrequent (12/215 [6%]) but consistent with the concept that, in some humans, BLV could be replicating.

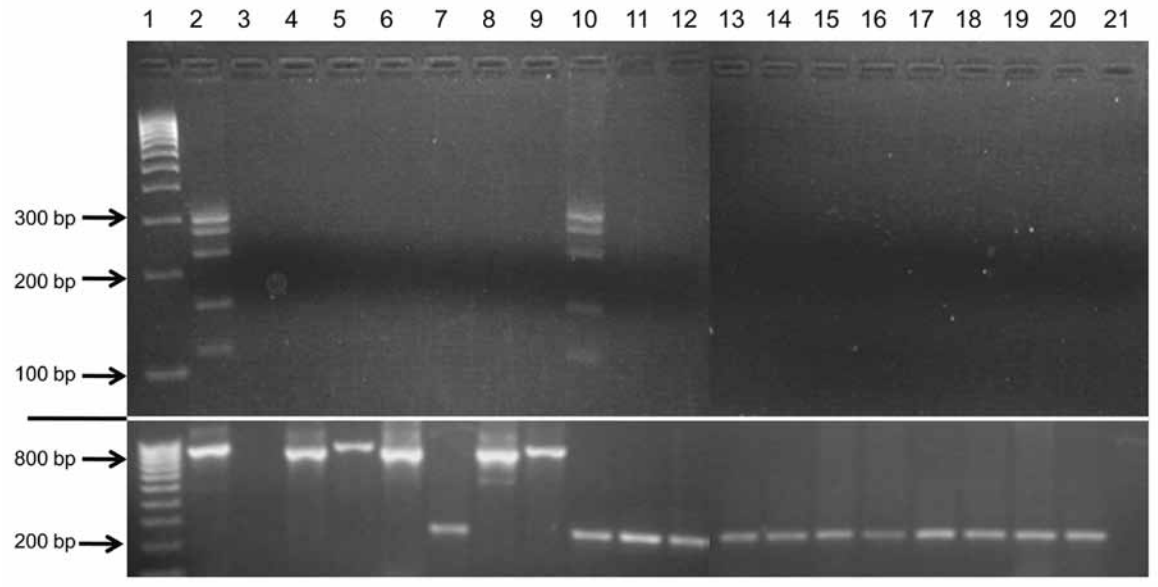


Figure 4. Test results showing lack of cross-reactivity of bovine leukemia virus (BLV)–specific primers with representatives of all mammalian and avian retrovirus subfamilies and human exogenous and endogenous viruses previously identified in human breast tissue. Nested liquid-phase PCR used primers from 5 BLV genome regions with template DNA from the viruses in lanes 4–10 and 12–21. PCR products for each virus, loaded into 1 well, were separated by agarose gel (1.5%) electrophoresis on the basis of size differences. Amplicons were generated only for known BLV-positive cell lines (FLK and Bat_2Cl_6). Samples in lanes 13–21 were run simultaneously in the same gel in wells below samples in lanes 4–12. The section below the white line shows glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification of each sample to indicate DNA quality. Human GAPDH primers were used for human, rhesus monkey, baboon, and bat cell lines (amplicon = 237 bp); murine GAPDH for mouse and rat cell lines (796 bp); and bovine GAPDH for bovine, ovine, and feline cell lines (857 bp). Lane 1, molecular weight marker (HyperLadder IV; Bioline, Taunton, MA, USA), lane 2, fetal lamb kidney cell line, positive control; lane 3, water substituted for DNA template, negative control; lane 4, Rous sarcoma virus; lane 5, murine sarcoma virus; lane 6, mouse mammary tumor virus; lane 7, Mason-Pfizer monkey virus; lane 8, murine leukemia virus; lane 9, feline leukemia virus; lane 10, BLV Bat_2Cl_6; lane 11, Tb_1Lu (known BLV-negative cell line), negative control; lane 12, simian T-cell leukemia virus; lane 13, human T-cell leukemia virus 1; lane 14, human T-cell leukemia virus 2; lane 15, HIV-1; lane 16, HIV-2; lane 17, human papillomavirus 16; lane 18, human papillomavirus 18; lane 19, Epstein-Barr virus; lane 20, human endogenous retrovirus K; lane 21, *env* of human endogenous retrovirus K.

Mutation rate tends to be lower for latent viruses than for viruses that escape immune attack by rapidly mutating to outpace the host's immune response (e.g., HIV [*2,31*]). Four of the 7 *LTR* base substitutions we observed (in 3 human BLV isolates) occurred in the PU box, a short (12 bp) protein-binding region that, when mutated, decreases basal gene expression (*2,33*). This expression would facilitate evasion of the host's immune response and would thus be likely to be selected for during virus evolution. Transcription in BLV-infected bovine lymphocytes is rare, identified in only ≈1/50,000 peripheral blood cells (*2*).

In summary, multiple lines of evidence support the conclusion that the BLV DNA and protein we found are more likely to represent the in vivo presence of BLV in humans than to represent some other virus, molecular laboratory contamination, or an artifactual nonspecific reaction. In view of the potential public health implications of BLV in humans, future research should address how humans acquire BLV infection, how frequently BLV infection occurs in different populations, and whether the virus is associated with human disease.

Acknowledgments

We thank Gary Firestone for providing the GR cell line; Kathryn Radke for the FLK, Bat_2Cl_6, and Tb_1Lu cell lines; Carl Hanson for the formalin-fixed HIV- and HTLV-infected cells; Feng Wang-Johanning for the HERV-K DNA; and Craig Meyers for the formalin-fixed keratinocyte raft cultures. We also thank Dr Firestone and Fenyong Liu for their critical reviews of the manuscript and Christina Di Loreto and Michael Van Ness for assistance with photography.

This research was supported by the following grants: California Breast Cancer Research Program of the University of California (6PB-0075); US Army Medical Research and Materiel Command Breast Cancer Research Program (DAMD 17-02-1-0320); and grants from the Dr. Susan Love Research Foundation and the Avon Foundation.

Dr Buehring is professor of virology in the Infectious Diseases and Vaccinology Division, School of Public Health, University of California, Berkeley. Her research interest is BLV, including its presence in the mammary gland of cows, hormone responsiveness to glucocorticoids, and the effects of its infection in humans.

References

1. Schwartz I, Levy D. Pathobiology of bovine leukemia virus. Vet Res. 1994;25:521–36.
2. Gillet N, Florins A, Boxus M, Burteau C, Nigro A, Vandermeers F, et al. Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in humans. Retrovirology. 2007;4:18–50. http://dx.doi.org/10.1186/1742-4690-4-18
3. US Department of Agriculture Animal and Plant Health Services. Info sheet: bovine leukosis virus (BLV) in U.S. beef cattle. February 1999 [cited 2013 Jan 23]. http://www.aphis.usda.gov/animal_health/nahms/beefcowcalf/downloads/beef97/Beef97_is_BLV.pdf
4. US Department of Agriculture Animal and Plant Health Services, Veterinary Services, Center for Epidemiology and Animal Health. Info sheet: bovine leukosis virus (BLV) on U.S. dairy operations, 2007. October 2008 [cited 2013 Aug 20]. http://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_is_BLV.pdf
5. Burridge MJ. The zoonotic potential of bovine leukemia virus. Vet Res Commun. 1981;5:117–26. http://dx.doi.org/10.1007/BF02214976
6. Choi KY, Liu RB, Buehring GC. Relative sensitivity and specificity of agar gel immunodiffusion, enzyme immunosorbent assay, and immunoblotting for detection of anti-bovine leukemia virus antibodies in cattle. J Virol Methods. 2002;104:33–9. http://dx.doi.org/10.1016/S0166-0934(02)00040-X
7. Buehring GC, Philpott SM, Choi KY. Humans have antibodies reactive with bovine leukemia virus. AIDS Res Hum Retroviruses. 2003;19:1105–13. http://dx.doi.org/10.1089/088922203771881202
8. Baumgartener L, Olson C, Onuma M. Effect of pasteurization and heat treatment on bovine leukemia virus. J Am Vet Med Assoc. 1976;169:1189–91.
9. Chung YS, Prior HC, Duffy PF, Rogers RJ, Macenzie AR. The effect of pasteurization on bovine leucosis virus–infected milk. Aust Vet J. 1986;63:379–80. http://dx.doi.org/10.1111/j.1751-0813.1986.tb02908.x
10. Buehring GC, Kramme PM, Schultz RD. Evidence for bovine leukemia virus in the mammary epithelial cells of infected cows. Lab Invest. 1994;71:359–65.
11. Cooper JK, Sykes G, King S, Cottril K, Ivanova NV, Hanner R, et al. Species identification in cell culture: a two-pronged molecular approach. In Vitro Cell Dev Biol Anim. 2007;43:344–51. http://dx.doi.org/10.1007/s11626-007-9060-2
12. Motton DD, Buehring GC. Bovine leukemia virus alters growth properties and casein synthesis in mammary epithelial cells. J Dairy Sci. 2003;86:2826–38. http://dx.doi.org/10.3168/jds.S0022-0302(03)73880-6
13. Ercolani L, Florence B, Denaro M, Alexander M. Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene. J Biol Chem. 1988;263:15335–41.
14. Gillio-Tos A, De Marco L, Fiano V, Garcia-Bragado F, Dikshit R, Boffetta P, et al. Efficient DNA extraction from 25-year-old paraffin-embedded tissues: study of 365 samples. Pathology. 2007;39:345–8. http://dx.doi.org/10.1080/00313020701329757
15. Nuovo GJ. PCR in situ hybridization: protocols and applications. Philadelphia: Lippincott-Raven; 1996. pp. 193–244.
16. Kramme PM, Thomas CB, Schultz RD. Temporal stability of the virus load of cattle infected with bovine leukemia virus. Vet Immunol Immunopathol. 1995;45:347–54. http://dx.doi.org/10.1016/0165-2427(94)05352-S
17. Joshi D, Buehring GC. Are viruses associated with breast cancer? Scrutinizing the molecular evidence. Breast Cancer Res Treat. 2012;135:1–15. http://dx.doi.org/10.1007/s10549-011-1921-4
18. Ressang AA. Examination for evidence of infection of humans with bovine leukemia virus [in Dutch]. Tijdschr Diergeneeskd. 1977;102:333–5.
19. Olson C, Driscoll DM. Bovine leukosis: investigation of risk for man. J Am Vet Med Assoc. 1978;173:1470–2.
20. Kettmann R, Burny A, Cleuter Y, Ghysdael J, Mammerickx M. Distribution of bovine leukemia virus proviral sequences in tissues of bovine, ovine, and human origin. Ann Rech Vet. 1978;9:837–44.
21. Thiry L, Sprecher-Goldberger S, Jacquemin P, Cogniaux J, Burny A, Portetelle D, et al. Bovine leukemia virus–related antigens in lymphocyte cultures infected with AIDS-associated viruses. Science. 1985;227:1482–4. http://dx.doi.org/10.1126/science.2579433

22. Bender AP, Robison LL, Kashmiri SV, McClain K, Woods WG, Smithson WA, et al. No involvement of bovine leukemia virus in childhood acute lymphoblastic leukemia and non-Hodgkin's lymphoma. Cancer Res. 1988;48:2919–22.
23. Chen IS, Haislip AM, Myers LW, Ellison GW, Merrrill JE. Failure to detect human T-cell leukemia virus–related sequences in multiple sclerosis blood. Arch Neurol. 1990;47:1064–5. http://dx.doi.org/10.1001/archneur.1990.00530100026008
24. Heneine W, Woods TC, Sinha SD, Khan AS, Chapman LE, Schonberger LB, et al. Lack of evidence for infection with known human and animal retroviruses in patients with chronic fatigue syndrome. Clin Infect Dis. 1994;18(Suppl 1):S121–5. http://dx.doi.org/10.1093/clinids/18.Supplement_1.S121
25. Perzova RN, Loughran TP, Dube S, Ferrer J, Esteban E, Poiesz BJ. Lack of BLV and PTLV DNA sequences in the majority of patients with large granular lymphocyte leukaemia. Br J Haematol. 2000;109:64–70. http://dx.doi.org/10.1046/j.1365-2141.2000.01972.x
26. Lee J, Kim Y, Kang CS, Cho DH, Shin DH, Yum YN, et al. Investigation of the bovine leukemia virus proviral DNA in human leukemia and lung cancers in Korea. J Korean Med Sci. 2005;20:603–6. http://dx.doi.org/10.3346/jkms.2005.20.4.603
27. Burmeister T, Schwartz S, Hummel M, Hoelzer D, Thiel E. No genetic evidence for involvement of Deltaretroviruses in adult patients with precursor and mature T-cell neoplasms. Retrovirology. 2007;4:11–7. http://dx.doi.org/10.1186/1742-4690-4-11
28. Christou L. The global burden of bacterial and zoonotic infections. Clin Microbiol Infect. 2011;17:326–30. http://dx.doi.org/10.1111/j.1469-0691.2010.03441.x
29. Diglio CA, Ferrer JF. Induction of syncytia by the bovine C-type leukemia virus. Cancer Res. 1976;36:1056–67.
30. Kamihira S, Sugahara K, Tsuruda K, Uemura A, Akamatsu N, Nagai H, et al. Proviral status of HTLV-1 integrated into the host genomic DNA of adult T-cell leukemia cells. Clin Lab Haematol. 2005;27:235–41. http://dx.doi.org/10.1111/j.1365-2257.2005.00698.x
31. Duffy S, Shackleton LA, Holmes EC. Rates of evolutionary change in viruses: pattern and determinants. Nat Rev Genet. 2008;9:267–76. http://dx.doi.org/10.1038/nrg2323
32. Igakura T, Stinchcombe JC, Goon PK, Taylor GP, Griffiths GM, Tanaka Y, et al. Spread of HTLV between lymphocytes by virus-induced polarization of the cytoskeleton. Science. 2003;299:1713–6. http://dx.doi.org/10.1126/science.1080115
33. Zhao X, Jimenez C, Sentsui H, Buehring GC. Sequence polymorphisms in the long terminal repeat of bovine leukemia virus: Evidence for selection pressures in regulatory sequences. Virus Res. 2007;124:113–24. http://dx.doi.org/10.1016/j.virusres.2006.10.010

Address for correspondence: Gertrude Case Buehring, Division of Infectious Diseases and Vaccinology, School of Public Health, 16 Barker Hall, University of California, Berkeley, CA 94720-7354, USA: email: buehring@berkeley.edu

Trends in Infectious Disease Mortality Rates, Spain, 1980–2011

Teresa López-Cuadrado, Alicia Llácer, Rocio Palmera-Suárez, Diana Gómez-Barroso, Camelia Savulescu, Paloma González-Yuste, and Rafael Fernández-Cuenca

Using mortality data from National Institute of Statistics in Spain, we analyzed trends of infectious disease mortality rates in Spain during 1980–2011 to provide information on surveillance and control of infectious diseases. During the study period, 628,673 infectious disease–related deaths occurred, the annual change in the mortality rate was –1.6%, and the average infectious disease mortality rate was 48.5 deaths/100,000 population. Although the beginning of HIV/AIDS epidemic led to an increased mortality rate, a decreased rate was observed by the end of the twentieth century. By codes from the International Classification of Diseases, 9th revision, the most frequent underlying cause of death was pneumonia. Emergence and reemergence of infectious diseases continue to be public health problems despite reduced mortality rates produced by various interventions. Therefore, surveillance and control systems should be reinforced with a goal of providing reliable data for useful decision making.

Although infectious diseases continue to account for considerable illness and death worldwide (*1,2*), mortality rates for these diseases in industrialized countries decreased considerably by end of the twentieth century (*1–4*). The decrease in infectious disease mortality rates was caused by a set of complex factors fundamentally linked to development, such as better sanitation as populations became more urban, and improvements in infrastructure, nutrition, and biotechnological advances, particularly in the field of vaccines and antimicrobial drugs (*5*). This reduction was reflected mainly in the decrease of child mortality rates. At the beginning of the twentieth century, 30% of all deaths caused by infectious diseases were among children <5 years of age; by the end of the twentieth century, these diseases accounted for only 1.4% of all deaths (*3*). In the 1980s, the decreasing trend of the infectious disease mortality rate in industrialized countries was interrupted by the HIV/AIDS epidemic, which confronted the scientific community and health authorities with the challenge of a new emerging infection that has still not been controlled.

In Spain, as in other industrialized countries, mortality rates decreased overall and for children over the course of the twentieth century, life expectancy increased dramatically. The progressive decrease in deaths from infectious causes was interrupted by the HIV/AIDS epidemic, which changed the trend and pattern of infectious diseases for the population overall and for specific age groups affected (*6*). New antiretroviral therapies introduced in the mid-1990s decreased deaths caused by HIV/AIDS, as well as deaths caused by other infectious diseases. However, other threats to human health related to emergence and reemergence of infectious disease have arisen (*7*), mainly because of environmental and climate changes, travel and trade, human behavior, new technologies, microbial adaptation, and host-impaired immunity (*8*). These continuous threats make specific infectious disease surveillance and control programs even more necessary (*8*).

All-cause and cause-specific mortality rates, as well as standardized mortality rates, are still good indicators for ascertaining the public health effects of a given disease and assessing trends in incidence. Successive revisions of the International Classification of Diseases (ICD) have continued to apply an etiologic criterion to pool part of infectious and parasitic diseases in a single group and leaving conditions of infectious origin in other groups. Pinner et al. (*9*) found that ICD codes for infectious and parasitic diseases in the ICD, 9th revision (ICD-9) included only 67% of the 1,131 codes that could be included as infectious diseases or consequences of infections, and applied comprehensive criteria to the analysis of infectious disease mortality rates. In Spain, infectious disease mortality rates in the early 1990s were assessed by using similar criteria and resulted in a

Authors' affiliations: Carlos III Institute of Health, Madrid, Spain (T. López-Cuadrado, A. Llácer, R. Palmera-Suárez, D. Gómez-Barroso, C. Savulescu, P. González-Yuste, R. Fernández-Cuenca); Centros de Investigación Biomédica en Red de Epidemiología y Salud Pública, Madrid (A. Llácer, R. Palmera-Suárez, D. Gómez-Barroso, R. Fernández-Cuenca); and Complejo Hospitalario de Toledo, Toledo, Spain (P. Gónzalez-Yuste)

DOI: http://dx.doi.org/10.3201/eid2005.131528

3-fold increase in number of deaths related to ICD codes for infectious and parasitic diseases (*6,10*). The purposes of the current study were to determine the magnitude of infectious disease mortality rates rate overall and by sex, age, and the principal causes implicated, and to describe trends during 1980–2011 to clarify surveillance needs and enhance control strategies.

Methods

In Spain, the source of mortality rate statistics is the medical death certificate, a compulsory administrative document that is completed by the physician who certifies the death. Data are subsequently forwarded to the regional mortality registries where causes of death are coded according to ICD guidelines. According to World Health Organization recommendations, the cause of death that is ICD coded should be taken as the underlying cause of death (*11*).

We analyzed ICD codes of underlying causes of death provided by the Spanish National Statistics Institute (NSI). We selected deaths caused by infectious causes corresponding to ICD-9 codes for 1980–1998 and ICD-10 codes for 1999–2011. From the ICD-9 and ICD-10 codes, we selected all codes of infectious and parasitic diseases and other infectious causes from remaining groupings (Table 1). Of these diseases, 10 that accounted for >90% of all deaths (pneumonia, septicemia, cardiac infections, AIDS and HIV infection, renal infections, tuberculosis, acute respiratory infection, influenza, viral hepatitis, and intestinal infections) were independently selected. For HIV/AIDS, data collection began in 1989 when a newly created specific ICD-9 code was assigned and began to be used in Spain; related diseases were previously allocated to unspecified immunity disorders.

We calculated the crude mortality rates by using population data drawn from NSI population projections. Age- and sex-adjusted rates were calculated by using the direct method and the standard European population as a reference.

We analyzed the trends according to sex, age group (<1–4, 5–24, 25–44, 45–64, and ≥65 years), and for each of the 10 first-selected diseases. We computed the sex ratio of the adjusted rates to assess sex-related differences. Subsequently, we analyzed the trends of death rates by using a joinpoint regression model to estimate the annual percentage change (APC) and to identify trend inflection points (joinpoints) when present. An inflection point was defined as the year representing the final endpoint of 1 period and the initial endpoint of a subsequent period; thus, all periods overlap. For each period, an APC was calculated.

We computed APC for each trend by using generalized linear models and assuming a Poisson distribution (*12*). This analysis initially assumes that there are no joinpoints and iteratively fits models until a curve with the minimum number of joinpoints is selected by using permutation tests (*13*). Adjusted rates and SEs were used to fit all joinpoint models, except for analyses by age group, for which deaths and populations under a Poisson model were used. This method identifies through simulations the minimum number

Table 1. Infectious diseases analyzed, Spain, 1980–2011*

Diseases/infections	ICD-9 codes	ICD-10 codes	No. cases		SR,	APC, % ,
			1980	2011 (%)	2007–2011	1980–2011
All causes			288,426	386,017		
Infectious diseases	NA	NA	19,106	22,646 (100)	1.5	–1.6†
Pneumonia	480–486	J12–J18	9,292	8,138 (35.9)	1.9	–2.9†
Sepsis	038	A40–A41	861	2,955 (13.0)	1.4	1.5†
Cardiac	390–398, 420–422	I00–I09, I30–I33, I40	2,702	2,213 (9.8)	0.9	–2.5†
AIDS and HIV	279.5, 279.6, 795.8	B20–B24, R75	NA	944 (4.2)	3.9	NA
Renal/urinary	590, 595, 599.0	N10–N12, N13.6, N15.1, N30, N39.0	474	3,565 (15.7)	1.1	3.1†
Tuberculosis and sequelae	010–018, 137	A15–A19, B90	1,475	284 (1.3)	3.0	–7.0†
Acute respiratory	460–466, 475, 510, 513, 034.0	J00–J08, J20–J22, J36, J85, J86	913	1,136 (5.0)	1.2	–1.6†
Influenza	487	J09–J11	997	214 (0.9)	1.2	–8.0†
Viral hepatitis	070	B15–B19	109	865 (3.8)	1.5	5.7†
Intestinal	001–009	A00–A09	402	719 (3.2)	1.0	–0.6
Other codes						
Infectious and parasitic diseases	020–033, 034.1, 035–037, 039–057, 060–066, 071–136, 138, 139	A20–A39, A42–A99, B00–B14, B25–B89, B91–B99	1,088	586 (2.6)	1.6	–2.9†
Other infectious diseases	320–323, 540–542, 566, 567.0–2, 569.5, 576.1, 770.0, 771	G00–G04, K35–K37, K61.0–4, K63.0, K65.0, K65.8, K67, K83.0, P23, P35–P39	793	1,027 (4.5)	1.4	–0.3

*ICD-9, International Classification of Diseases, 9th revision; ICD-10, International Classification of Diseases, 10th revision; SR, sex ratio (M:F, age-adjusted rate); APC, annual percentage change (age-adjusted rate); NA, not applicable.
†Significant because APC CIs did not include 0.

of inflection points (i.e., years when the trend is changing) and quantifies changes in trends through the APC. When the APC is positive and significant, it indicates that the trend is increasing. When the APC is negative and significant, it indicates that the trend is decreasing.

Statistical significance was set at $p<0.05$. Data analyses were performed by using Stata 12 (StataCorp. LP, College Station, TX, USA) and Joinpoint Regression version 3.5.1 software (http://surveillance.cancer.gov/joinpoint/Joinpoint_Help_4.0.4.pdf). This study was conducted as an activity of infectious disease surveillance at the National Center of Epidemiology, Madrid, Spain.

Results

During 1980–2011, there were 628,673 deaths caused by infectious diseases in Spain. Although the crude mortality rate decreased from 50.9 to 49.2 deaths/100,000 persons during this period, the adjusted mortality rate decreased from 53.8 to 27.3 deaths/100,000 persons. The joinpoint method identified 2 inflection points in the trend, the first in 1987 and the second in 1994. Adjusted mortality rates indicated a decrease during 1980–1987 and an APC of –6.2% (95% CI –8.5% to –3.9%), an increase during 1987–1994 and an APC of 3.1% (95% CI –0.1% to 6.4%); and a decrease during 1994–2011 and an APC of –2.5% (95% CI –3.1% to –1.9%).

Among men, 4 periods of change were observed. The first period (1980–1987) had an APC of –5.9% (95% CI –8.1% to –3.6%), the second period (1987–1995) had an APC of a 4.9% (95% CI 2.4%–7.2%), the third period (1995–1998) had an APC of –7.8% (95% CI –20.6% to 7.6%), and fourth period (1998–2011) had an APC of –2.5% (95% CI –3.4% to –1.7%). Changes in first, second, and fourth periods were significant because the APC CIs did not include 0.

Among women, only 2 periods (1980–1986 and 1986–2011) of change were observed. Both of these changes were significant and showed decreases (APC –6.3%, 95% CI –9.0% to –3.4% and APC –1.0%, 95% CI –1.4% to –0.7%); the inflection point was in 1986 (Figure 1). During 2007–2011, the male:female sex ratio was 1.5:1 for deaths caused by infectious diseases and was higher for 9 of the 10 diseases first selected, except for cardiac infections (Table 1).

There were major variations in mortality rates by age group and sex; children <1–4 years of age and persons ≥65 years of age showed the largest shifts. Sex-specific mortality rates were higher for male patients across all age groups. Although the largest decrease in deaths caused by infectious disease was among children <1–4 years of age (47.4 deaths/100,000 persons in 1980 and 8.4 deaths/100,000 persons in 2011), the lowest rates were observed among persons 5–24 years of age. A notable peak in deaths occurred in 1989–1997 because of AIDS; the population segment most affected was men 25–44 years of age (Figure 2). The study period showed a major decrease in mortality rates among male and female patients across all age groups. The sharpest decrease was among persons <1–4 years of age, who had an APC of –5.5% (95% CI –6.2% to –4.8%) for male patients and –5.0% (95% CI –5.6% to –4.4%) for female patients. However, among persons >65 years of age, this decrease was much smaller (-0.5%, 95% CI –0.8% to –0.1% for men and –0.1%, 95% CI –0.3% to 0.6% for women) (Table 2).

Influenza, pneumonia, acute respiratory infection, and septicemia accounted for 58% of the infectious diseases studied. The mortality rate for pneumonia showed the

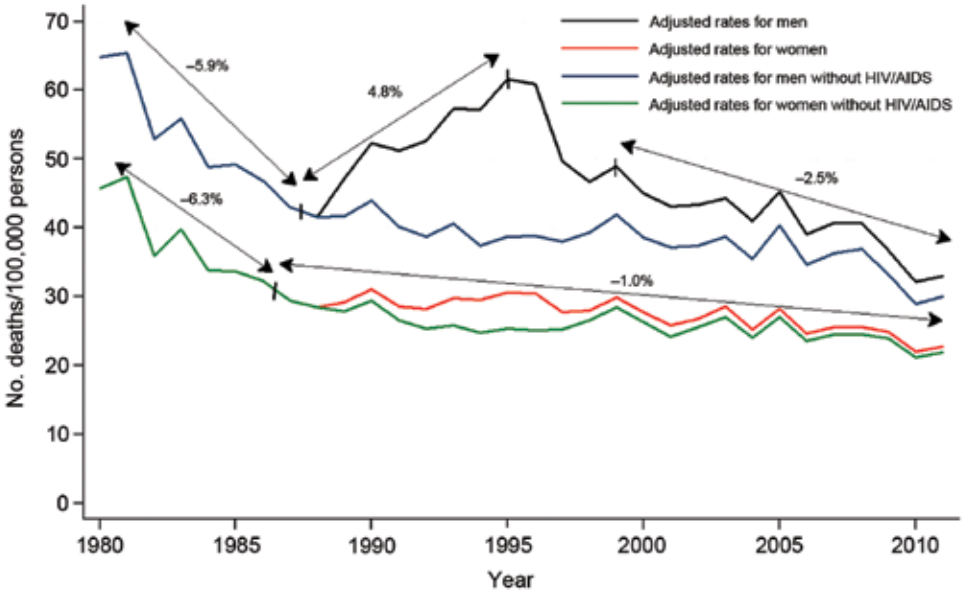


Figure 1. Infectious disease mortality rates by sex, Spain, 1980–2011.

largest decrease. Starting in 1997, deaths caused by tuberculosis and HIV/AIDS decreased across the study period. Deaths caused by cardiac and renal infections showed mutually opposite trends: deaths caused by cardiac infections decreased gradually over time, and deaths caused by renal infections increased over time. Viral hepatitis and intestinal infections had the lowest mortality rates (Figure 3).

Discussion

Our results showed that mortality rates for infectious diseases in Spain decreased overall and that these decreases were only temporarily interrupted by the HIV/AIDS epidemic. Sex-specific mortality rates were higher for male patients across all age groups. Of the 10 first studied diseases, pneumonia showed the largest decrease in mortality rate.

Previous studies showed that analysis of infectious disease mortality rates should take into account infectious disease–related ICD codes from groups other than infectious and parasitic diseases in different ICD revisions because relying only on infectious and parasitic diseases will lead to underestimation of infectious disease mortality rates (*1,6,10,14,15*). Using this approach, we showed that 3 times more deaths were caused by infectious disease in Spain in 2011 than when only codes for infectious and parasitic diseases were analyzed, which resulted in a reported underestimated rate (*16*). Our estimated rate was 27.3 deaths/100,000 persons when adjusted for age, with a 1.5-fold increased mortality rate for male patients compared with female patients. Thus, diseases in other ICD diagnostic groups would account for 6% of the general mortality rate, and infectious diseases would be the fourth most common cause of death, instead of the eleventh most common cause of death, if only codes for infectious and parasitic diseases were considered.

Comparison of mortality rates for Spain with those for other countries in Europe is difficult because no standardized data are available, whether because such data solely take the traditional categories into account or because they refer to different periods. For 2010, Eurostat data (*17*) for the European Union (27 member states) showed an adjusted mortality rate of 8.7 deaths/100,000 persons for infectious and parasitic diseases and a male:female sex ratio of 1.7:1.

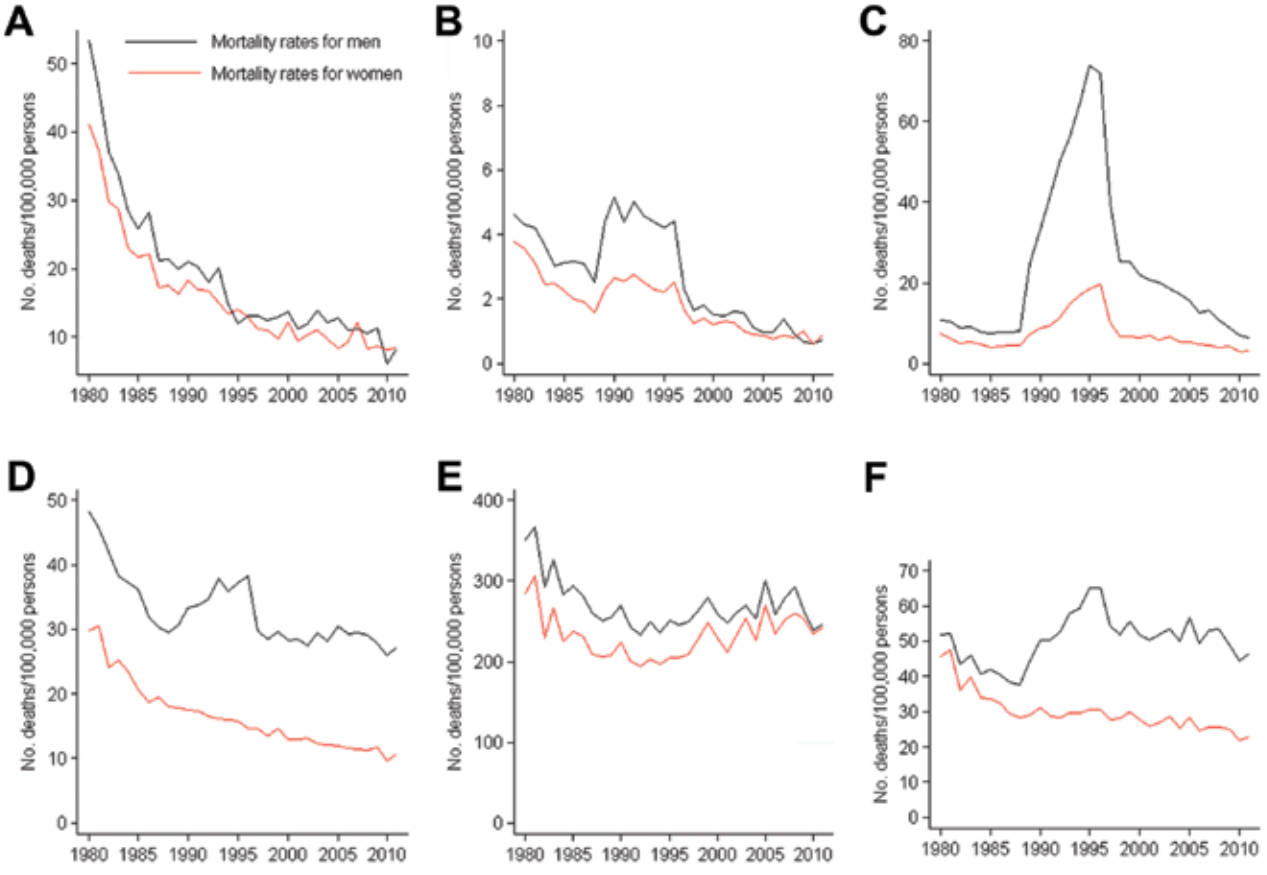


Figure 2. Infectious disease mortality rates by sex and age group, Spain, 1980–2011. A) <1–4 y, B) 5–24 y, C) 25–44 y, D) 45–64 y, E) ≥65 y, F) all ages.

Table 2. Mortality rates for infectious diseases analyzed, by sex, age group, and detected trends, Spain, 1980–2011*

Sex/age group, y	Rate for 1980	Rate for 2011	APC for 1980–2011, %	Trend 1		Trend 2		Trend 3		Trend 4	
				Period	APC, %	Period	APC, %	Period	APC, %	Period	APC, %
M											
<1–4	53.3	8.3	−5.5†	1980–1985	−14.2†	1985–2011	−4.0†	–	–	–	–
5–24	4.6	0.7	−4.2†	1980–1986	−7.7†	1986–1992	10.7†	1992–2011	−11.15†		
25–44	10.7	6.4	−2.8	1980–1985	−4.8	1985–1995	27.0†	1995–1998	−31.3†	1998–2011	−9.7†
45–64	48.1	27.2	−1.4†	1980–1987	−6.7†	1987–1995	3.3†	1995–1998	−8.8	1998–2011	−0.3
≥65	350.2	247.1	−0.5†	1980–1991	−3.3†	1991–2008	1.0†	2008–2011	−5.4	–	–
AR	64.9	33.0	−1.5†	1980–1987	−5.9†	1987–1995	4.8†	1995–1998	−7.7	1998–2011	−2.5†
F											
<1–4	41.1	8.5	−5.0†	1980–1987	−10.9†	1987–2011	−3.3†				
5–24	3.8	0.9	−4.4†	1980–1988	−9.4†	1988–1991	19.1	1991–2011	−7.3†	–	–
25–44	7.4	3.1	−1.7	1980–1986	−9.9†	1986–1995	21.3†	1995–1998	−28.2†	1998–2011	−6.3†
45–64	29.9	10.8	−3.2†	1980–1986	−7.6†	1986–2011	−2.5†	–	–	–	–
≥65	284.0	242.5	−0.1	1980–1989	−4.0†	1989–2011	1.2†	–	–	–	–
AR	45.7	22.70	−1.6†	1980–1986	−6.3†	1986–2011	−1.0†	–	–	–	–

*Rates are no. cases/100,000 persons. APC, annual percentage change (age-adjusted or age-specific rate); –, not applicable; AR, age-adjusted rates.
†Significant because APC CIs did not include 0.

Our finding of higher mortality rates for male patients for nearly all infectious diseases analyzed, in particular for HIV/AIDS and tuberculosis, was consistent with higher illness rates for male patients because of biologic or behavioral factors (*15,18*). In general, these sex-related differences were similar to those for other countries in Europe (*17*).

The HIV/AIDS epidemic in Spain interrupted the decreasing trend of deaths caused by infectious disease in the late 1980s. The initial increase in the HIV/AIDS epidemic was caused mainly by use of shared needles and other items related to injection drug use (*19*). The efficacy of parenteral transmission caused this infection to spread rapidly, and during 1988–1997, Spain had the highest incidence of AIDS in Europe (*20*); the highest incidence was in 1994 (*21*). In 1996, highly active antiretroviral therapy (*21*) was introduced in Spain; it became widely available and was dispensed free of charge. This new treatment resulted in major changes in infectious disease trends in Spain and in mortality rates for AIDS in specific age groups.

In the age group most affected by HIV (persons 25–64 years of age), the imbalance in infection rates between men and women resulted in different trends in infectious disease mortality rates; these mortality rates for men have not yet returned to pre-AIDS rates. For persons 35–44 years of age, infectious diseases accounted for 8% of all deaths in 2011 and were the most frequent cause of death after suicides. For men 25–34 years of age, the mortality rate for other causes has become a more critical issue than the mortality rate for infectious diseases.

In the youngest and oldest age groups, infectious disease mortality rates showed similar trends for both sexes. Children <1–4 years of age showed the sharpest continuous decrease, although this trend has shown a gradual leveling in the past 20 years, which is consistent with low mortality rates for children already observed (*22,23*). However, infectious diseases still account for 12% of all deaths in this age group, a finding consistent with reports from other countries (*24,25*).

Persons ≥65 years of age were the only age group for whom infectious disease mortality rates increased after the early 1990s, in contrast to a previously decreasing trend (*10*). In this age group, 5 infectious diseases accounted for 4%–7% of all deaths. This trend was essentially caused by deaths of persons in the oldest age group (>80 years), whose relative effect on this trend has increased because of an increase in life expectancy (82 years in 2011). Currently, persons >65 years of age make up 17% of the population of Spain. Of these persons, 14% are >85 years of age. In a health system that provides universal coverage, hospitalization of this frequently immunodeficient elderly population increases the risk for infection. Therefore, septicemia and renal infections are the main causes of the increase in infectious disease mortality rates, as reported for other countries (*26*). However, the 3

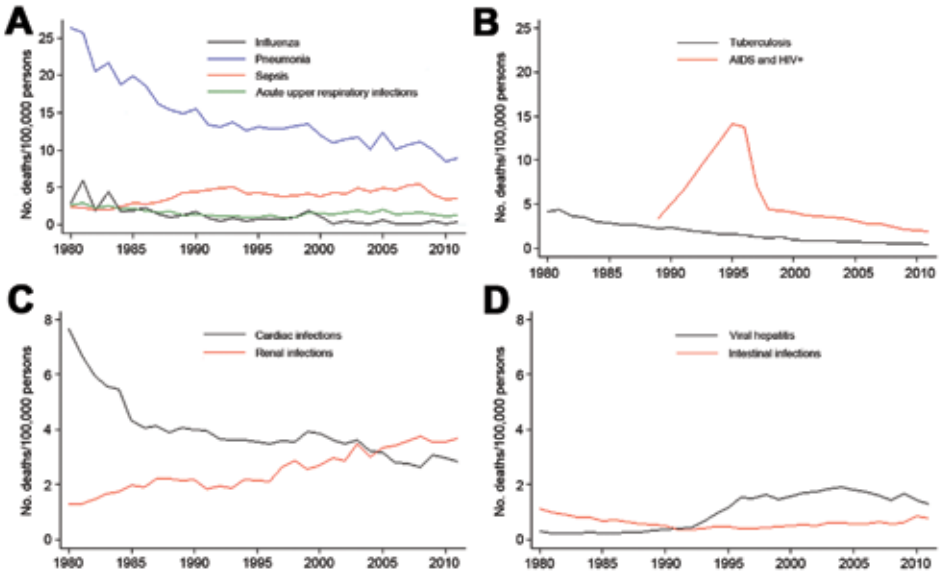


Figure 3. Mortality rates for selected infectious diseases, Spain, 1980–2011.

other causes (pneumonia, cardiac infections, and respiratory infections), which along with those mentioned, accounted for 90% of the infectious disease mortality rate in elderly persons, have had no role in this increase.

In addition to the increase in septicemia and renal infections, attention should be given to deaths caused by viral hepatitis, a disease that initially showed low mortality rates. However, these rates have increased 9-fold over the past 20 years. This cause of death, which accounted for 4% of deaths caused by infectious diseases, had the highest APC for the study period, and its increase has been especially evident among persons >45 years of age, in particular, those >65 years of age. The increase in deaths caused by viral hepatitis has partly paralleled the AIDS epidemic because both diseases have similar transmission mechanisms. The incidence of hepatitis C has increased in Europe and that of hepatitis B has generally decreased (*27*), probably because of vaccination.

Among relevant infectious respiratory diseases at the beginning of the study period, only pneumonia retained its role as the leading infectious cause of death. Deaths caused by respiratory tuberculosis were surpassed by deaths caused by septicemia, a disease that accounted for 16.6% of deaths caused by infectious diseases. Tuberculosis mortality rates showed a decreasing trend because of lower incidence and efficacy of treatment. AIDS initially led to a reemergence of tuberculosis but has since tended to mask this reemergence, probably because AIDS has being increasingly listed as the underlying cause of death on death certificates.

This study had 3 principal limitations. First, cause-specific death statistics in Spain include only the primary underlying cause of death (*11*), which omit possible contributions of other infectious causes and result in underestimation of deaths caused by infectious diseases, a finding that could be detected only by using multiple coding (*28*). Second, in 1998 Spain replaced ICD-9 with ICD-10, a decision that might have been led to problems of standardization of disease coding and discontinuity in assessment of trends. Third, we were not able to include factors such as improvements in diagnosis and medical practices.

In conclusion, despite a decrease in deaths caused by infectious diseases that was interrupted by HIV/AIDS, infectious diseases continue to be a major cause of death, which is an indicator of their role in public health. In the twenty-first century, the incidence of such diseases might be increased by a series of factors (*7,29*), ranging from climate change, which alters the ecology of vectors, to globalization, which involves exchange of goods and mobility of persons for leisure, occupational, or survival purposes. Notable among these factors is excessive use of antimicrobial drugs (*30*) among humans and animals. This excessive use is a fundamental cause of increases in bacterial drug resistance and is responsible for 25,000 deaths annually in Europe (*31*). Newly resistant or multidrug-resistant bacterial strains are more lethal, and emerging or reemerging diseases caused by these strains would be more difficult to control.

In addition to the aforementioned factors, the economic crisis in Europe has resulted in a decrease in health expenditures; public health services have been the most affected most by budget cuts (*32*). Among these services, prevention programs, such as disease surveillance and control systems that target populations at risk for infectious diseases, have been greatly affected by underfunding (*32*). In this situation, information provided by vital statistics complements these services for retrospective assessment of trends of infectious disease mortality rates in Spain (*33*).

Acknowledgments

We thank the HIV Surveillance Unit of the Institute of Health Carlos III, especially Asunción Diaz, for providing comments on this article.

Dr López-Cuadrado is a researcher at the National Center for Epidemiology of the Institute of Health Carlos III, Madrid, Spain. Her primary research interest is epidemiology of infectious diseases and statistical methods to investigate them.

References

1. Armstrong GL, Conn LA, Pinner RW. Trends in infectious disease mortality in the United States during the 20th century. JAMA. 1999;281:61–6. http://dx.doi.org/10.1001/jama.281.1.61
2. Bi P, Whitby M, Walker S, Parton KA. Trends in mortality rates for infectious and parasitic diseases in Australia: 1907–1997. Intern Med J. 2003;33:152–62. http://dx.doi.org/10.1046/j.1445-5994.2003.00354.x
3. Centers for Disease Control and Prevention. Achievements in public health: control of infectious diseases. MMWR Morb Mortal Wkly Rep. 1999;48:621–9.
4. Arkwright PD, David TJ. Past mortality from infectious diseases and current burden of allergic diseases in England and Wales. Epidemiol Infect. 2005;133:979–84.. http://dx.doi.org/10.1017/S0950268805004516
5. Communicable diseases. Health status of Spaniards [in Spanish]. Madrid: Ministerio de Sanidad y Consumo; 1998.
6. Martínez de Aragón MV, Llácer A, Martínez Navarro JF. Infectious disease mortality in Spain: 1980–1995 [in Spanish]. Boletin Epidemiológico Semanal. 1998;6:165–72.
7. 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.. http://dx.doi.org/10.1038/nature06536
8. MacLehose L, Mckee M, Weinberg J. Responding to the challenge of communicable disease in Europe. Science. 2002;295:2047–50. http://dx.doi.org/10.1126/science.1070025
9. Pinner RW, Teutsch SM, Simonsen L, Klug LA, Graber JM, Clarke MJ, et al. Trends in infectious diseases mortality in the United States. JAMA. 1996;275:189–93. http://dx.doi.org/10.1001/jama.1996.03530270029027
10. Fernández de la Hoz K, de Mateo S, Regidor E. Trends in infectious diseases mortality in Spain [in Spanish]. Gac Sanit. 1999;13:256–62.
11. National Statistics Institute. Methodology of death statistics according to cause of death [cited 2013 Jun 29]. http://www.ine.es/daco/daco42/sanitarias/notaecm.htm
12. Kim HJ, Fay MP, Feuer EJ, Midthune DN. Permutation tests for joinpoint regression with applications to cancer rates. Stat Med. 2000;19:335–51. http://dx.doi.org/10.1002/(SICI)1097-0258(20000215)19:3<335::AID-SIM336>3.0.CO;2-Z
13. Clegg LX, Hankey BF, Tiwari R, Feuer EJ, Edwards BK. Estimating average annual per cent change in trend analysis. Stat Med. 2009;28:3670–82. http://dx.doi.org/10.1002/sim.3733
14. Serraino D, Bidoli E, Piselli P, Angeletti C, Bruzzone S, Pappagallo M, et al. Time trends in infectious disease mortality in Italy: 1969–1999 [in Italian]. Epidemiol Prev. 2004;28:322–9.
15. Shohat T, Harari G, Green MS. Mortality from infectious diseases in Israel, 1979–1992, based on revised ICD-9 codes: implications for international comparisons. Am J Public Health. 1999;89:1855–7. http://dx.doi.org/10.2105/AJPH.89.12.1855
16. National Statistics Institute. Mortality and cause of deaths, 2011 [in Spanish] [cited 2013 Feb 27]. http://www.ine.es/prensa/np767.pdf
17. European Commision. Populations and social conditions [cited 2013 Jun 30]. http://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=hlth_cd_asdr&lang=en
18. Owens IP. Ecology and evolution. Sex differences in mortality rate. Science. 2002;297:2008–9. http://dx.doi.org/10.1126/science.1076813
19. Díez M, Oliva J, Sanchez F, Vives N, Cevallos C, Izquierdo A. Incidence of new HIV diagnoses in Spain, 2004–2009 [in Spanish]. Gac Sanit. 2012;26:107–15.
20. European Centre for the Epidemiological Monitoring of AIDS. HIV/AIDS surveillance in Europe. Mid-year report, no. 63. St. Maurice (France): The Center; 2001 [cited 2014 Feb 12]. http://www.ceses.org/AidsSurv/rapport_n63_2000/rapport63eng.htm
21. Secretary of the National AIDS Strategy. HIV/AIDS in Spain. Epidemiological situation in 2001 [in Spanish]. Madrid: Ministry of Health; 2002.
22. Llácer A, Fernández-Cuenca R, Pérez B. Change in the infant mortality in Spain in the last twenty years [in Spanish]. Boletin Epidemiológico Semanal. 2004;23:257–60.
23. DiLiberti JH, Jackson CR. Long-term trends in childhood infectious disease mortality rates. Am J Public Health. 1999;89:1883–5. http://dx.doi.org/10.2105/AJPH.89.12.1883
24. Read JS, Troendle JF, Klebanoff MA. Infectious disease mortality among infants in the United States, 1983 through 1987. Am J Public Health. 1997;87:192–8. http://dx.doi.org/10.2105/AJPH.87.2.192
25. Wilson D, Bhopal R. Impact of infection on mortality and hospitalization in the North East of England. J Public Health Med. 1998;20:386–95. http://dx.doi.org/10.1093/oxfordjournals.pubmed.a024792
26. Angeletti C, Piselli P, Bidoli E, Bruzzone S, Puro V, Girardi E, et al. Analysis of infectious disease mortality in Italy [in Italian]. Infez Med. 2004;12:174–80.
27. Rantala M, van de Laar MJ. Surveillance and epidemiology of hepatitis B and C in Europe—a review. Euro Surveill. 2008;13: pii: 1880.
28. García Benavides F, Godoy C, Perez S, Bolumar F. Multiple codification of the causes of death: from dying "of" to dying "from" [in Spanish]. Gac Sanit. 1992;6:53–7. http://dx.doi.org/10.1016/S0213-9111(92)71092-9
29. Fears R, ter Meulen V. Health benefits of policies to mitigate climate change. Lancet. 2011;377:995–6. http://dx.doi.org/10.1016/S0140-6736(11)60385-1
30. Carlet J, Collignon P, Goldmann D, Goossens H, Gyssens IC, Harbarth S, et al. Society's failure to protect a precious resource: antibiotics. Lancet. 2011;378:369–71. http://dx.doi.org/10.1016/S0140-6736(11)60401-7
31. European Centre for Disease Prevention and Control. The bacterial challenge: time to react [cited 2013 Jun 30]. http://www.ecdc.europa.eu/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf
32. Semenza JC, Tsolova S, Lim TA. Economic crisis and infectious disease control: a public health predicament. Eur J Public Health. 2012;22:5–6. http://dx.doi.org/10.1093/eurpub/ckr212
33. Rechel B, Suhrcke M, Tsolova S, Suk JE, Desai M, Mckee M, et al. Economic crisis and communicable disease control in Europe: a scoping study among national experts. Health Policy. 2011;103:168–75. http://dx.doi.org/10.1016/j.healthpol.2011.06.013

Address for correspondence: Teresa López-Cuadrado, National Center for Epidemiology, Carlos III Health Institute, Av. Monforte de Lemos 5, 28029 Madrid, Spain; e-mail:teresalc@isciii.es

Molecular Characterization of Cryptically Circulating Rabies Virus from Ferret Badgers, Taiwan

Hue-Ying Chiou, Chia-Hung Hsieh, Chian-Ren Jeng, Fang-Tse Chan, Hurng-Yi Wang,[1] and Victor Fei Pang[1]

After the last reported cases of rabies in a human in 1959 and a nonhuman animal in 1961, Taiwan was considered free from rabies. However, during 2012–2013, an outbreak occurred among ferret badgers in Taiwan. To examine the origin of this virus strain, we sequenced 3 complete genomes and acquired multiple rabies virus (RABV) nucleoprotein and glycoprotein sequences. Phylogeographic analyses demonstrated that the RABV affecting the Taiwan ferret badgers (RABV-TWFB) is a distinct lineage within the group of lineages from Asia and that it has been differentiated from its closest lineages, China I (including isolates from Chinese ferret badgers) and the Philippines, 158–210 years ago. The most recent common ancestor of RABV-TWFB originated 91–113 years ago. Our findings indicate that RABV could be cryptically circulating in the environment. An understanding of the underlying mechanism might shed light on the complex interaction between RABV and its host.

Rabies is possibly one of the oldest zoonotic diseases. It is caused by the rabies virus (RABV), a neurotropic virus in the family *Rhabdoviridae*, genus *Lyssavirus*. Except for a small number of countries and regions, particularly islands, RABV is found worldwide. The virus infects nearly all warm-blooded animals and causes severe neurologic signs, which almost invariably lead to death (*1*). It was estimated that worldwide in 2010, the disease caused >60,000 human deaths, primarily in Africa and Asia (*2*). Although dogs are considered the principal host of RABV in developing countries, the virus is also dispersed among many species of wild carnivora and chiroptera, especially in those countries of Europe and North America that have well-established vaccination programs (*3*). Mustelids, including various species of the genera *Melogale*, *Meles*, and *Mellivora* of the weasel family Mustelidae, can carry RABV (*4–6*). In southeastern China, Chinese ferret badgers (CNFB; *Melogale moschata moschata*) have been associated with human rabies for many years and are considered to be a primary host in this region (*7–9*).

After what were considered to be the last reported cases of rabies in a human and a nonhuman animal in 1959 and 1961, respectively, Taiwan was rabies free for >50 years until the 2012–2013 outbreak of ferret badger–associated rabies. During May 2012–January 2013, through a government-supported program of routine disease surveillance of free-range dead wild animals that had been killed by vehicles or were receiving treatment for injuries and/or illness at the wildlife first aid station, 3 dead Taiwan ferret badgers (TWFB; *M. moschata subaurantiaca*) were submitted to the School of Veterinary Medicine, National Taiwan University, for further examination.

Pathologic examination revealed nonsuppurative meningoencephalomyelitis with formation of eosinophilic intracytoplasmic inclusion bodies in all 3 animals; reverse transcription PCR and immunohistochemical staining excluded the possibility of infection with the canine distemper virus. However, the results of fluorescence antibody testing, immunohistochemical staining, and reverse transcription PCR, followed by sequencing for RABV, were positive (H.-Y. Chiou, unpub. data). After the rabies diagnoses for the initial 3 ferret badgers were confirmed, by the end of August of 2013, rabies had been diagnosed by fluorescence antibody testing for an additional 105 dead or ill and euthanized ferret badgers and 1 shrew.

Author affiliations: National Taiwan University, Taipei, Taiwan, Republic of China (H.-Y. Chiou, C.-H. Hsieh, C.-R. Jeng, H.-Y. Wang, V.F. Pang); and Council of Agriculture, Executive Yuan, Nantou County, Taiwan, Republic of China (F.-T. Chan)

DOI: http://dx.doi.org/10.3201/eid2005.131389

[1]Joint senior authors who contributed equally to this article.

Our objective in this study was to clarify whether the current outbreak of the TWFB–associated rabies is an emerging, a reemerging, or a cryptically circulating disease. We investigated the possible origin of this outbreak and its relations with CNFB-associated rabies in mainland China via genomic organization and characterization and analysis of genetic diversity and phylogeographic origin of RABV-TWFB. In addition, we propose a mechanism that might be contributing to the limited host range of RABV-TWFB.

Materials and Methods

Animals and Specimen Collection

During May 2012–January 2013, three ill TWFB were collected from different regions of central Taiwan (Figure 1). One was in the Xitou nature education area at Lugu Township, Nantou County (R2012–26); one was in Gukeng Township, Yunlin County (R2012–88); and one was in Yuchih Township, Nantou County (R2013–01). These 3 TWFB, respectively, showed the following clinical signs: emaciation, coma, paddling, loss of pain response, reduced body temperature, and a 2-cm skin wound on the chin; extreme weakness and inability to move; and signs of weakness and respiratory signs, including labored breathing and increased breath sounds with hypersalivation and exudation of foamy fluid from the mouth and nose. Initial supportive treatment was provided at the wildlife first aid station, but the ferret badgers died within 1–3 days, and their carcasses were submitted to the School of Veterinary Medicine, National Taiwan University, for routine disease surveillance. Full necropsy was performed, during which half of the left cerebral hemisphere was collected from each animal and stored at –80°C for subsequent nucleic acid extraction. Representative tissue samples were taken from all major organs and fixed in 10% neutral buffered formalin for histopathologic examination.

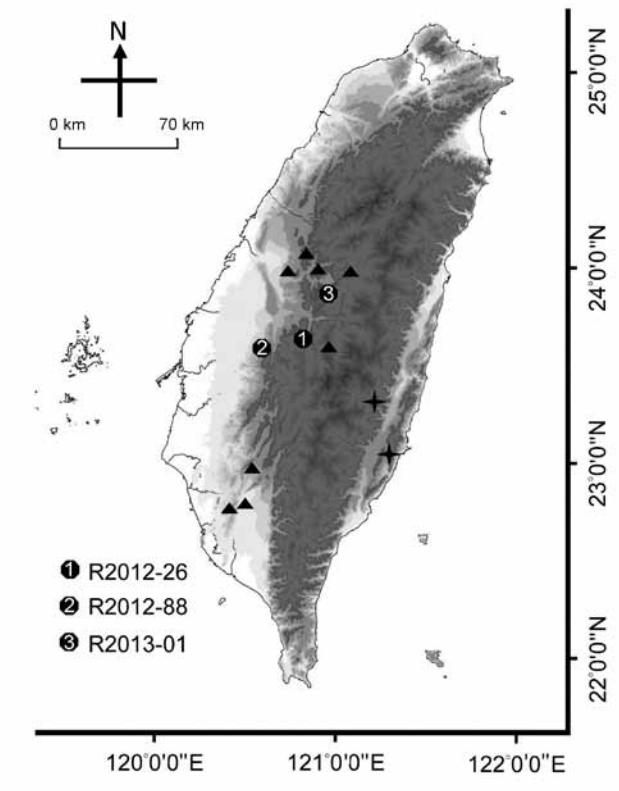


Figure 1. Collection sites of rabies-positive Taiwan ferret badgers (TWFB), Taiwan. Solid circles marked with 1–3 represent the collection sites of the first 3 rabies-positive animals. Triangles represent the collection sites of other rabies virus (RABV) sequences included in this study. Crosses represent the most diverged lineages of rabies virus from Taiwan ferret badgers (TWFB, TW1614, and TW1955), shown in Figure 5, panel B, Appendix (wwwnc.cdc.gov/EID/article/20/5/13-1389-F5.htm), and the easternmost cross represents the isolate from a shrew, TW1955.

Sample Preparation and Genome Sequencing

Approximately 25 mg of brain specimen from each animal was homogenized, and 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added. Total RNA was extracted by using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), and cDNA was synthesized by using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. To amplify the whole genome, we used 19 pairs of primers (Table 1), including the forward primer for the 5′ end and the reverse primer for the 3′ end designed to be complementary to the respective ends of the genome, as described (*10*).

Sequence Analyses and Phylogenetic Reconstruction

Sequences were assembled by using the Seqman program (Lasergene 8, Madison, WI, USA) (GenBank accession nos. KF620487–KF620489) and then aligned by using the ClustalW program (*11*). The genetic distance was estimated by using the Kimura 2-parameter substitution model implemented in MEGA version 5.0 (*12*). The nucleotide diversity within populations was calculated by using DnaSP version 5.0 (*13*). To test for the deviation of neutral expectation, we conducted the Tajima D (*14*) and the Fu and Li D* (*15*) tests implemented in DnaSP. Significance was assessed by 10^4 coalescent simulations (*13*).

To investigate the phylogenetic position of RABV-TWFB isolates, we included 24 complete RABV genomes representing the 3 major phylogenetic groups (*16*). For global phylogeny of RABV, we analyzed 218 full-length (1,335-nt) sequences of the nucleoprotein (N) gene, including 11 sequences from Taiwan. We also analyzed 125 full-length (1,575-nt) sequences of the glycoprotein (G) gene, including 13 sequences from Taiwan (*17*). For each gene, phylogenetic trees were inferred by using maximum-likelihood and Bayesian inference methods.

Table 1. Primers used for the amplification and sequencing of rabies virus genome*

Primer	Sequence, 5′→3′	Amplicon size, nt	Position, nt
3′F	GTACCTAGACGCTTAACAAC	499	1–499
3′R	AAGACCGACTAAGGACGCAT		
NF	ATGTAACACCTCTACAATGG	1,533	55–1587
NR	CAGTCTCYTCNGCCATCT		
PF	GAACCAYCCCAAAYATGAG	1,001	1500–2500
PR	TTCATTTTRTYAGTGGTGTTGC		
MF	AAAAACRGGCAACACCACT	641	2479–3119
MR	TCCTCYAGAGGTAWACAAGTG		
G1F	TGGTGTATAACATGRAYTC	1,097	3000–4096
G1R	ACCCATGTYCCRTCATAAG		
G2F	TGGATTTGTGGATKAAAGAGGC	1,542	3995–5536
G2R	GAGTTNAGRTTGTARTCAGAG		
L1F	TGGRGAGGTYTATGATGACCC	726	5430–6155
L1R	CAGCATNAGTGTRTAGTTTCTGTC		
L2F	GGTCGATTATGATAAKGCATTTGG	704	5885–6588
L2R	TTGACAGACCCTTTCGATAATC		
L3F	GGATCAATTCGACAACATACATG	550	6473–7024
L3R	AAGTCTTCATCHGGCARTCCTCC		
L4F	AGACTAGCTTCHTGGYTGTCAG	708	6882–7589
L4R	TACTTTGGTTCTTGTGTTCCTG		
L5F	AGTGTTTGGATTGAAGAGAGTGTT	662	7337–7998
L5R	GAAAGACTGCCTGCACTGACAT		
L6F	AATAGTCAACCTCGCCAATATAATG	767	7897–8645
L6R	GGATCTCTGAGTTGTAGAAGGATTC		
L7F	CCGAGTCAATCATTGGATTGATAC	621	8517–9137
L7R	GAATACCCTCCTTCGCTGTATCTG		
L8F	GAGAAGGTCACCAATGTTGATG	1,045	8958–10002
L8R	AGATCCAYARCCAGTCATTCTC		
L9F	ACATAATGCTCAGAGAACCGT	503	9820–10322
L9R	CCATTCTGAACATCCTACCTT		
L10F	TGTTCAGAATGGGTCTGCTCT	509	10302–10811
L10R	TGCATCGCAAATAATGAGGT		
L11F	ATTATTTGCGATGCAGAAGT	524	10797–11320
L11R	ATGATAGCCACTTTAGACAGAGT		
L12F	GTTACAGAGGGGAACTCTGTCT	386	11285–11670
L12R	TCTTCACTATCTTGTAAATCAACCT		
5′F	TGGATCAGGTTGATTTACAAGATAGT	293	11640–11932
5′R	ACGCTTAACAAATAAACAACAAAAAT		

*Primers were from Lei et al. (*10*).

The maximum-likelihood analysis was conducted by using PhyML 3.0 online (*18*); the starting tree was derived from the neighbor-joining method, and the nearest neighbor interchange topology search option was used. The nucleotide substitution model for phylogenetic reconstruction was determined by using the Akaike information criterion implemented in jModeltest 0.1.1 (*19*). The method of Bayesian inference was performed by using MrBayes version 3.1.2 (*20*). Analyses were initiated with random starting trees, and Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run for 1×10^6 generations and sampled every 100 generations. The steady state of the log-likelihood was reached at ≈20,000 generations. Subsequently, the first 201 trees were excluded and the remaining 9,800 trees were retained to compute a 50% majority-rule consensus tree.

Divergence Dating

The divergence time between different viral lineages and the time to the most recent common ancestor (TMRCA) of virus isolates were estimated by using an established Bayesian MCMC approach implemented in BEAST version 1.7 (*21*). The analysis was performed by using the general time-reversible model of nucleotide substitution assuming an uncorrelated lognormal molecular clock (*22*). We linked substitution rates for the first and second codon positions and allowed independent rates in the third codon position. The molecular clocks were 2.3×10^{-4} (range $1.1–3.6 \times 10^{-4}$) and 3.9×10^{-4} ($1.2–6.5 \times 10^{-4}$) substitutions/site/year for N and G genes, respectively (*17*). A slightly faster clock, 4.3×10^{-4} ($3.1–5.6 \times 10^{-4}$) substitutions/site/year for N gene (*23*), was also used in a separate analysis.

Because a previous study revealed that the population dynamics of RABV supported a model of constant population size through time (*17*), we restricted our analysis to this demographic model. For each analysis, we performed 2 independent runs with 2×10^7 MCMC steps, of which the first 10% were discarded as burn-in. To confirm that both were sampling the same distribution, we compared and then combined the results. Log files were checked by

using Tracer (http://beast.bio.ed.ac.uk/Tracer), and the effective sample size for each parameter was >300, which is adequate according to the authors of BEAST software.

Results

Genomic Organization and Characterization of RABV-TWFB

Similar to previous analyses (*16*,*17*), our phylogenetic analysis that used whole RABV genomes revealed 3 major groups with high bootstrap support (Figure 2). Although the 3 RABV-TWFB isolates are clustered within the Asia group, composed of 3 distinct lineages, (China I [including CNFB], China II [*16*], and Southeast Asia), they do not appear close to any of the 3 lineages. More noteworthy, the 3 isolates of RABV-TWFB are not close to those of RABV-CNFB, indicating that they may have originated independently.

The genome of RABV-TWFB is 11,923 nt long and encodes 5 proteins. The nucleotide lengths of different genomic regions are within the range of variations in different Asia lineages (Table 2), except the matrix protein (M)-G intergenic region, which is 1 nt longer than in the rest of the lineages from Asia (212 vs. 211). Within the group of Asia lineages, the most conserved protein is M, followed by N, the virion-associated RNA polymerase (L), and G; the least conserved is phosphoprotein (P) (Table 3). Among the RABV groups, however, N becomes the most conserved followed by L, M, G, and P. The RABV-TWFB is closest to China I lineage in the N, P, and L gene regions, but it is closest to RABV-CNFB in the M and G gene regions.

The genetic variations across the whole genome among different lineages can be viewed in a sliding window analysis (Figure 3). Within N, there seems to be a conserved central domain previously identified in RABV at residues 182–328 (*24*), which is also conserved in RABV-TWFB. The P, the last quarter of G and G–L intergenic regions, and the last part of L are more variable among different lineages of RABV than is the rest of the genome. The conserved M is functional in viral assembly and budding (*25*); is involved in the regulation of transcription and replication of viral RNA (*26*); and has been reported to induce apoptosis (*27*), suggesting its role in host-cell interplay. The involvement of M in multiple interactions explains its conservation among lineages. G is responsible for cell attachment and fusion and is the main viral protein responsible for the induction of neutralization antibodies and cell-mediated immune responses. The region between aa 189 and aa 214,

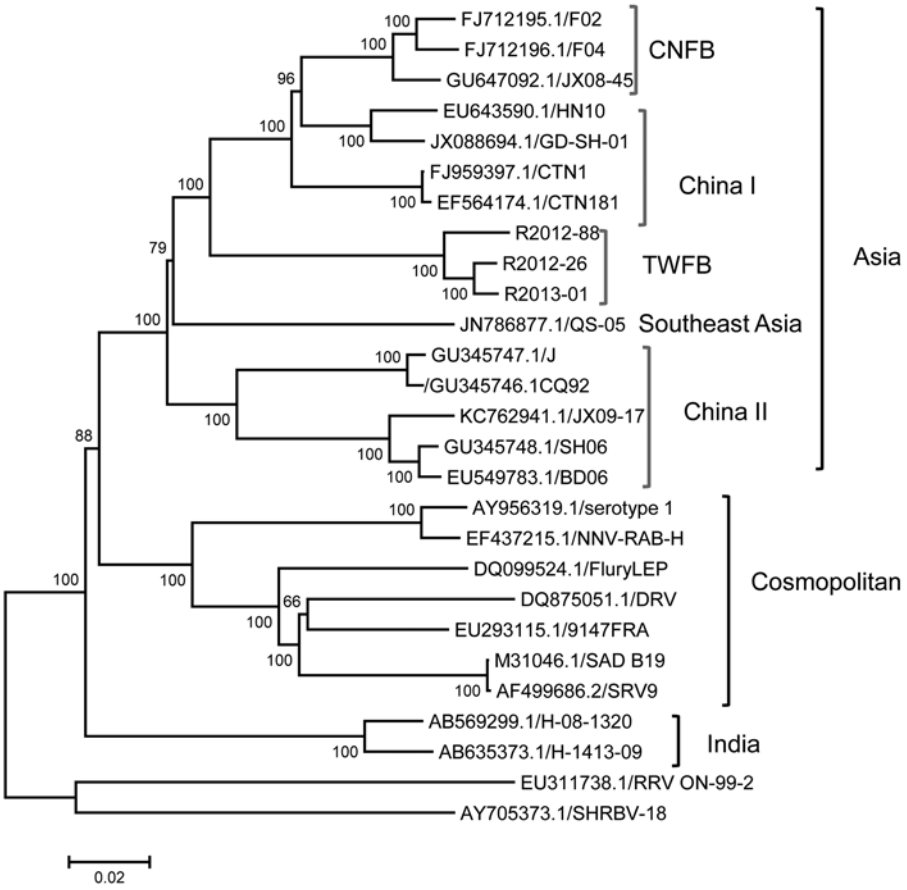


Figure 2. Phylogenetic relationships of 27 rabies virus (RABV) genomes constructed by maximum-likelihood method. Numbers close to the nodes were from 1,000 bootstrap replications. The tree was rooted with RABV from bats and raccoons. Three major groups, Asia, Cosmopolitan, and India, are strongly supported, as indicated (*17*). There are 4 major lineages within the group from Asia, including previously recognized China I, China II (*16*), Southeast Asia, and RABV from Taiwan ferret badgers (TWFB). RABVs derived from Chinese ferret badgers (CNFB) are clustered with China I, indicating that RABVs of TWFB and CNFB are of independent origin. Scale bar indicates nucleotide substitutions per site.

Table 2. Genomic organization and nucleotide lengths of terminal and intergenic regions and viral genes of rabies virus from Taiwan and other lineages from Asia*

Isolate	3′-UTR	Gene, nucleotide length									5′-UTR	Genome size
		N	N–P	P	P–M	M	M–G	G	G–L	L		
TWFB	70	1,353	91	894	86	609	212	1,575	519	6,384	130	11,923
CNFB	70	1,353	91	894	86	609	211	1,575	519	6,384	130–131	11,922–11,923
China I†	70	1,353	90–91	894	87–88	609	211	1,575	519	6,384	130	11,923
China II†	70	1,353	91	894	87	609	211	1,575	518–519	6,384	131	11,923–11,924

*UTR, untranslated region; TWFB, Taiwan ferret badgers; CNFB, Chinese ferret badgers.
†The definitions of China I and II are based on He et al. (16). China I does not include CNFB.

proposed to be needed for G binding to the nicotinic acetylcholine receptor (*28*), is relatively conserved in the dataset. Nevertheless, 3 substitutions (N194Y, R196K, and G203E) are found exclusively in RABV-TWFB G. In L, Poch et al. (*29*) recognized 6 conserved blocks, including B1 (233–424), B2 (504–608), B3 (609–832), B4 (890–1061), B5 (1091–1326), and B6 (1674–1749) (*29*). In addition, 2 regions, L1 (1418–1515) and L2 (1884–1961), are also conserved across lyssaviruses (*30*). In RABV-TWFB, the B4 and L1 regions in L are variable, and the rest of the blocks are conserved (Figure 3).

Genetic Diversity and Phylogeographic Origin of RABV-TWFB

The data shown in Figure 2 indicate that RABV-TWFB is a distinct lineage within the Asia group of viruses. To further explore the detailed origin of RABV-TWFB, we included the representative N and G sequences of RABV from human and various animal species for analysis (*17*,*31*). Because maximum-likelihood and Bayesian inference methods yielded similar topologies, we report only the results derived from the former. Both N and G gene trees support the conclusion that RABV-TWFB is a distinct lineage within the Asia group, clustered with the China I lineage, including RABV-CNFB, and sequences from the Philippines (Figure 4, Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1389-F4.htm).

Divergence time was estimated by using a Bayesian coalescent approach. In this analysis, we included only sequences of the Asia group. On the basis of the molecular clock of 4.3×10^{-4}/site/year for N gene (*23*), the substitution rate at the third codon position is 1.1×10^{-3}/site/year, and RABV-TWFB was separated from China I and the Philippines isolates 158 years ago with 95% highest posterior density (HPD) ranging from 110 to 225 years (Figure 5, panel A, Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1389-F5.htm). The divergence between China I and the Philippines isolates occurred 132 (95% HPD, 90–192) years ago, which is similar to previous estimations (*23*,*32*). The TMRCA of isolates from Taiwan was 91 years (95% HPD, 57–137). A similar timescale, with overlapping 95% HPD, was derived by using the molecular clock of 2.3×10^{-4}/site/year for N gene (*17*) (Figure 5, panel A, hatched numbers, Appendix). The mean substitution rate for G gene sequences was 3.5×10^{-4}/site/year (7.8×10^{-4} for the third codon position), and the divergence of RABV-TWFB, China I, and the Philippines isolates was initiated 210 (107–553) years ago, and the TMRCA of isolates from Taiwan was 113 (53–296) years (Figure 5, panel B, Appendix). It is notable that the TMRCA of RABV-TWFB was more ancient than that of several distinct lineages in Figure 5, Appendix. For example, the TMRCA was 62–116 years for the Southeast Asia lineage and 54–102 years for RABV of the Philippines. The origin of RABV-CNFB was relatively recent; TMRCA was 13–25 years.

The nucleotide diversities of RABV-TWFB are 3.14% for the N and 4.21% for the G genes (Table 4), which are almost 5 times higher than those of RABV-CNFB, which are 0.67% for the N and 0.87% for G genes. For comparison purposes, the 65 N and 232 G gene sequences of RABV isolates from the Philippines were also included for analysis (*33*). The nucleotide diversities are 2.00% for the N gene and 2.57% for the G gene. The results of both the Tajima D and the Fu and Li D* tests are not significant for

Table 3. Genetic distances between Taiwan isolates and other rabies virus lineages or groups in different genomic regions*

Rabies virus group†	Gene					
	N	P	M	G	L	Genome
Asia						
CNFB	0.115	0.172	0.105	0.129	0.130	0.134
China I‡	0.104	0.157	0.107	0.133	0.123	0.127
China II	0.130	0.186	0.132	0.172	0.142	0.152
Southeast Asia	0.140	0.189	0.125	0.169	0.147	0.155
Cosmopolitan	0.161	0.216	0.173	0.209	0.177	0.192
India	0.152	0.229	0.183	0.211	0.175	0.191
Outgroup	0.200	0.284	0.212	0.237	0.209	0.232

*CNFB, Chinese ferret badgers; outgroup, rabies virus derived from bat and raccoon.
†Groups of rabies virus are based on the work of Bourhy et al. (*17*).
‡In this analysis, China I does not include CNFB.

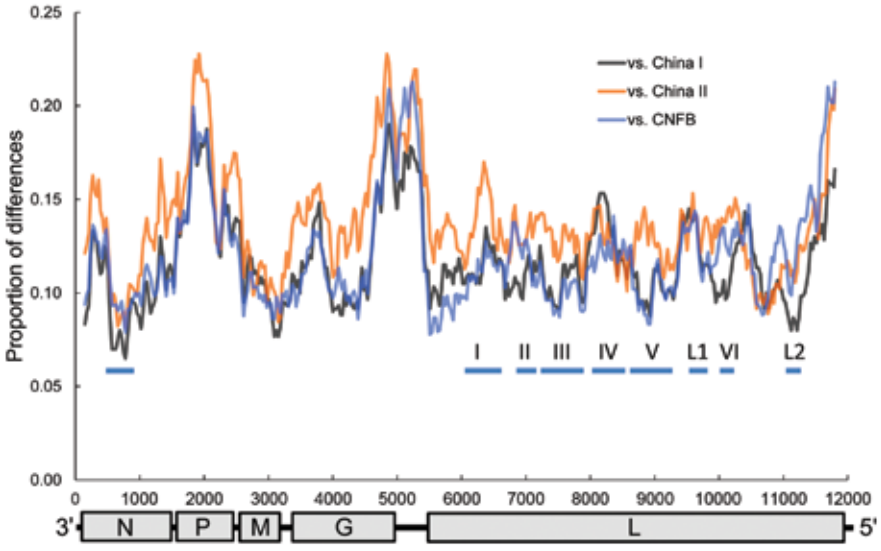


Figure 3. Sliding window analysis of rabies virus (RABV) genetic variations between Taiwan ferret badgers and China I, China II, and Chinese ferret badgers (CNFB). The genomic organization of RABV is shown at the bottom with nucleotide positions on the x-axis. The thick horizontal lines indicate conserved regions across lyssaviruses. N, nucleoprotein; P, phosphoprotein; M, matrix protein; G, glycoprotein; L, virion-associated RNA polymerase.

RABV-TWFB, indicating that the viral population is under neutral equilibrium, which in turn suggests that RABV was not recently introduced to TWFB. In contrast, the results of the Tajima D and the Fu and Li D* tests are significantly negative for the sequences of RABV-CNFB and sequences from the Philippines isolates, which are caused by an excessive of low-frequency mutation or by differentiation among populations.

Discussion

The Ancient Origin of RABV-TWFB

We sequenced and characterized a RABV strain, RABV-TWFB, recently isolated from ferret badgers in Taiwan. Our data showed that RABV-TWFB is clustered with sequences from the Philippines, China I, and RABV-CNFB. This relationship is strongly supported on the basis of multiple sequences of the N and G genes and of the complete genome (Figures 2 and 4, Appendix). Of ferret badger isolates, RABV-TWFB and RABV-CNFB come from phylogenetically distinct lineages, indicating that multiple RABV colonization events in this species probably occurred. A major question addressed in this study is whether RABV was recently introduced into the population of TWFB or perpetuated in TWFB without revealing its presence pathogenically after it was first introduced in the ancient past. Our divergence dating showed that the RABV has been circulating in TWFB for ≈100 years.

Our divergence and TMRCA estimations have a few potential sources of error. First, the RABV isolates from Taiwan might have originated from several introduction events, including the probability that multiple viral lineages occurred in the recent past and that the inflated TMRCA resulted from the combination of different, highly differentiated virus strains. Nevertheless, all isolates from Taiwan formed a monophyletic lineage distinct from other virus isolates. Unless several undetected virus strains were circulating around Taiwan, which is highly unlikely, the phylogenetic analyses support the existence of only 1 origin of RABV-TWFB.

Second, the ancient estimates could have resulted from the application of an inadequate molecular clock. However, the nucleotide substitution (mutation) rates of $2.3–4.3 \times 10^{-4}$ and 3.9×10^{-4}/site/year, for the N and G genes, respectively, used in the study reported here are in agreement with findings of other studies of lyssavirus evolution (*17*,*23*,*32*,*34*,*35*). In a study of RABV in bats, Streicker et al. (*34*) found that the nucleotide substitution rates in the third codon position, which are predominately silent (synonymous) substitutions, among viral lineages in different bat species spanned $8.3 \times 10^{-5}–2.1 \times 10^{-3}$/site/year. Our estimations of mutations of 1.1×10^{-3} and 7.8×10^{-4}/site/year for the third codon position of the N and G genes, respectively, are actually close to the upper boundary of their estimations. Therefore, our results should be conservative.

Third, RABV-TWFB exhibits high nucleotide diversity in the N and G genes. Notably, 232 G gene sequences collected from a large area of the Philippines showed nucleotide diversity that was two thirds that of RABV-TWFB. Taken together, all current genetic evidence supports the hypothesis of the ancient origin of RABV-TWFB. In addition, RABV-TWFB has been maintained in a large population for a long time.

Last, our recent retrospective study that used the archived formalin-fixed and paraffin-embedded brain tissues of ferret badgers, kindly provided by various institutes, demonstrated that the current earliest TWFB-associated

Table 4. Nucleotide diversity of rabies virus from TWFB, CNFB, and the Philippines*

Virus, gene	Length, nt	Sample size, no.	Π, %	Tajima D	Fu and Li D*
TWFB					
N	1,335	11	3.14	−0.886	0.735
G	1,575	12	4.21	−0.264	0.740
CNFB					
N	1,350	12	0.67	−1.294†	−1.918†
G	1,575	14	0.87	−1.168†	−1.336†
Philippines					
N	1,124	65	2.00	−1.380†	−1.759†
G	1,575	232	2.57	−1.676†	−3.80‡

*TWFB, Taiwan ferret badgers; CNFB, Chinese ferret badgers.
†p<0.05.
‡p<0.01.

RABV infection could be traced back to 2004 (H.-Y. Chiou, unpub. data), representing the oldest specimens that we have so far. That finding is consistent with the notion of a long history of RABV-TWFB in Taiwan.

Mutations in the G Gene of RABV-TWFB

The ancient history of RABV-TWFB raises 2 issues. First, because for the past 50 years Taiwan was believed to have been free from rabies, learning that the virus must have been cryptically circulating in the environment for such a long time is surprising. Because previous rabies surveillance was mainly focused on dogs and bats (www.baphiq.gov.tw), cases in remote areas might have gone unnoticed. However, Taiwan is an island with a high population density; 23 million persons live in an area of 36,188 km^2, and for rabies cases to have gone unnoticed for >50 years would be very unusual. Second, according to a recent survey about wildlife, the ferret badger population has been increasing in the past 5 years (L.-K. Lin, pers. comm.). Therefore, despite the ancient history of the ferret badger's association with RABV, the fact that its population is seemingly unaffected by infection with RABV is perplexing.

Except for 1 isolate from a shrew, all RABV isolates in the recent rabies outbreak in Taiwan have come from ferret badgers. The close relationship between the shrew RABV and ferret badger RABV collected from the same area suggests that the former probably resulted from spillover from the latter (Figure 5, panel B, Appendix). According to the most recent rabies surveillance data, the ferret badger is probably the only source of RABV in the current outbreak in Taiwan. Speculation that this RABV strain has adapted to and has been circulating in TWFB for a long time is reasonable. Its ability to transmit across species (e.g., ferret badger to shrew) is, thus, worthy of further investigation.

Among the multiple substitutions in RABV-TWFB genome that distinguish it from other virus strains, several substitutions in G (i.e., N194Y, R196K, and G203E) might merit additional attention. It has been demonstrated that a single amino acid mutation, N194K, in the nonpathogenic RABV vaccine strain SAD B19 was solely responsible for its increased pathogenicity. The increased pathogenicity is caused by increased virus spread in vivo and faster internalization of the virus into cells (*36*), both of which are consistent with the notion that G plays a major role in RABV pathogenesis. When the amino acid N was exchanged with amino acid S at position 194 (N194S), the pathogenic phenotype was reversed (*36*,*37*). Faster internalization of the virus into cells after N194K substitution might suggest that this region has some role in host cell binding. Evidence is strong that the muscular form of the nicotinic acetylcholine receptor, the neuronal cell adhesion molecule, and the p75 neurotrophin receptor serve as receptor sites for RABV binding and/or facilitate its entry into host cells (*38*,*39*). The 3 above-mentioned substitutions in RABV-TWFB are located in the region (aa 189 and 214) proposed to be needed for the binding of the G to the nicotinic acetylcholine receptor (*28*).

In our analysis of 113 G gene sequences from dog-associated RABV, no amino acid substitution was observed at the above-mentioned sites. In a total of 120 G gene sequences from bat-associated RABV (*40*), 8 N194T (6.7%), 2 N194S (1.7%), and 36 R196K (30.0%) amino acid substitutions were revealed with no amino acid change at G203. Taken together, the 3 aa substitutions (N194Y, R196K, and G203E) found in all 13 G gene sequences of RABV-TWFB are unique and worthy of further investigation.

Acknowledgments

We thank the Animal Health Research Institute, Council of Agriculture, Executive Yuan (ROC) for providing 8 and 10 full-length sequences of the N and G genes, respectively, for phylogenetic analysis. Special thanks go also to the Taiwan Endemic Species Research Institute, Council of Agriculture, for collection and submission of carcasses of free-range wildlife.

This research was supported in part by grants 101AS-10.1.2-BQ-B1(1) and 102AS-10.1.1-BQ-B1(1) from the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Executive Yuan, Taiwan (ROC) (to V.F.P.) and

NSC102-2313-B-002-062 from the Ministry of Science and Technology, Taiwan (ROC) (to H.-Y.W.).

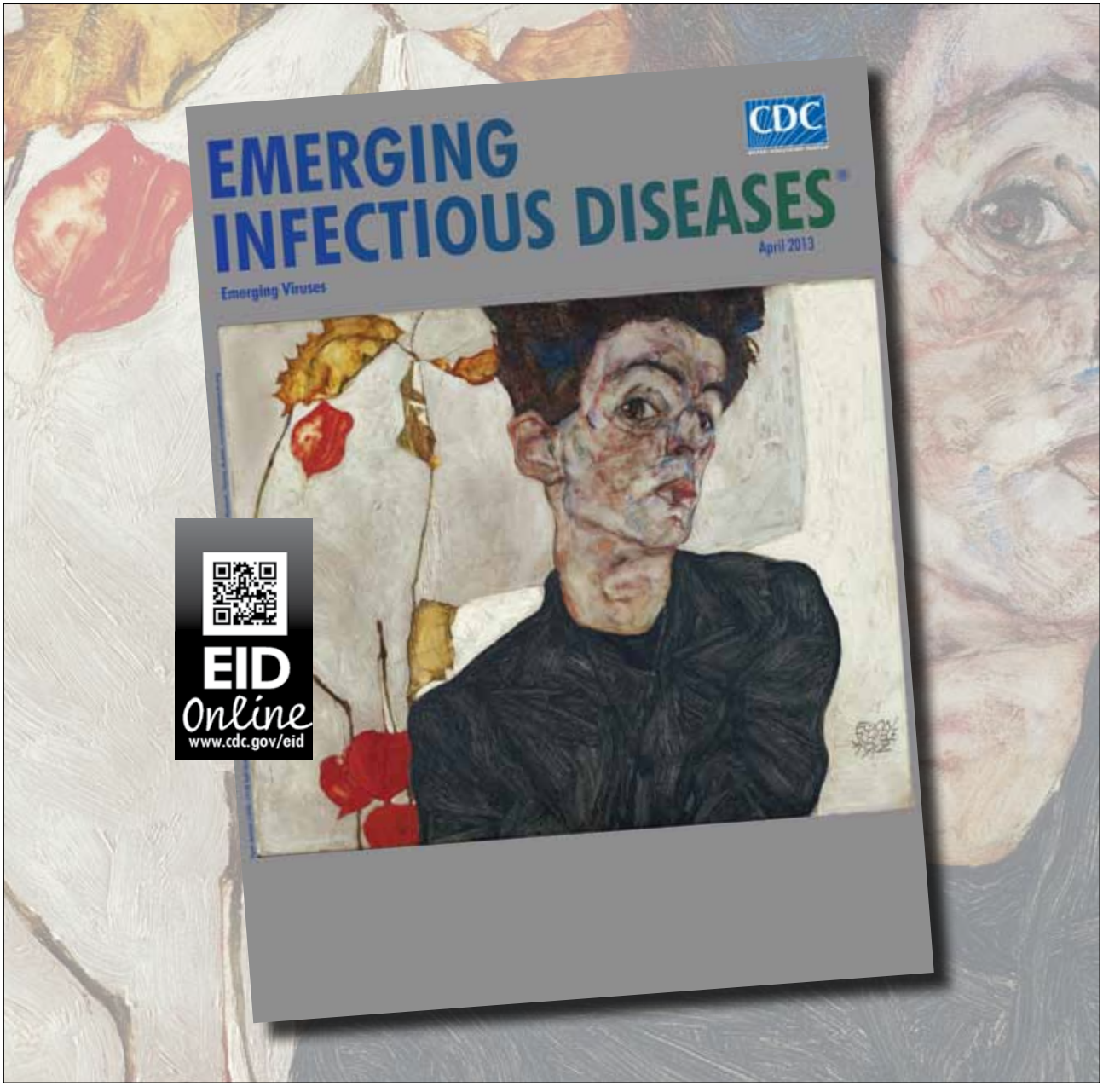
Dr Chiou is a chief pathology resident and a PhD candidate in the School of Veterinary Medicine, National Taiwan University. Her primary research interests include disease surveillance and monitoring of wildlife and zoonotic diseases.

References

1. Jackson AC. Pathogenesis. In: Jackson AC, Wunner WH, editors. Rabies. London: Elsevier Academic Press; 2007.
2. World Health Organization. WHO Expert Consultation on Rabies: second report. Geneva: The Organization; 2013. p. 1–139.
3. Wandeler A. Virus infections of non-domestic carnivores: rabies virus. In: Appel MJ, editor. Virus infections of carnivores. Amsterdam: Elsevier Science Publishers; 1987. p. 449–61.
4. Barnard BJH. The role played by wildlife in the epizootiology of rabies in South Africa and South-West Africa. Onderstepoort J Vet Res. 1979;46:155–63.
5. Smith GC, Wilkinson D. Modelling disease spread in a novel host: rabies in the European badger *Meles meles*. Journal of Applied Ecology. 2002;39:865–74. http://dx.doi.org/10.1046/j.1365-2664.2002.00773.x
6. Wandeler A, Wachendorfer G, Forster U, Krekel H, Muller J, Steck F. Rabies in wild carnivores in central Europe. II. Virological and serological examinations. Zentralbl Veterinarmed B. 1974;21:757–64. http://dx.doi.org/10.1111/j.1439-0450.1974.tb00479.x
7. Liu Y, Zhang SF, Wu XF, Zhao JH, Hou YL, Zhang F, et al. Ferret badger rabies origin and its revisited importance as potential source of rabies transmission in southeast China. BMC Infect Dis. 2010;10:234. http://dx.doi.org/10.1186/1471-2334-10-234
8. Zhenyu G, Zhen W, Enfu C, Fan H, Junfen L, Yixin L, et al. Human rabies cluster following badger bites, People's Republic of China. Emerg Infect Dis. 2007;13:1956–7. http://dx.doi.org/10.3201/eid1312.070465
9. Zhang S, Tang Q, Wu XF, Liu Y, Zhang F, Rupprecht CE, et al. Rabies in ferret badgers, southeastern China. Emerg Infect Dis. 2009;15:946–9. http://dx.doi.org/10.3201/eid1506.081485
10. Lei YL, Wang XG, Liu FM, Chen XY, Ye BF, Mei JH, et al. Complete genome sequencing and analyses of rabies viruses isolated from wild animals (Chinese ferret-badger) in Zhejiang Province [in Chinese]. Zhonghua Liu Xing Bing Xue Za Zhi. 2009;30:824–8.
11. 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. http://dx.doi.org/10.1093/nar/22.22.4673
12. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9. http://dx.doi.org/10.1093/molbev/msr121
13. Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009;25:1451–2. http://dx.doi.org/10.1093/bioinformatics/btp187
14. Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics. 1989;123:585–95.
15. Fu YX, Li WH. Statistical tests of neutrality of mutations. Genetics. 1993;133:693–709.
16. He CQ, Meng SL, Yan HY, Ding NZ, He HB, Yan JX, et al. Isolation and identification of a novel rabies virus lineage in China with natural recombinant nucleoprotein gene. PLoS ONE. 2012;7:e49992. http://dx.doi.org/10.1371/journal.pone.0049992
17. Bourhy H, Reynes JM, Dunham EJ, Dacheux L, Larrous F, Huong VT, et al. The origin and phylogeography of dog rabies virus. J Gen Virol. 2008;89:2673–81. http://dx.doi.org/10.1099/vir.0.2008/003913-0
18. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 2003;52:696–704. http://dx.doi.org/10.1080/10635150390235520
19. Posada D. jModelTest: phylogenetic model averaging. Mol Biol Evol. 2008;25:1253–6. http://dx.doi.org/10.1093/molbev/msn083
20. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572–4. http://dx.doi.org/10.1093/bioinformatics/btg180
21. Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol. 2012;29:1969–73. http://dx.doi.org/10.1093/molbev/mss075
22. Drummond AJ, Ho SY, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with confidence. PLoS Biol. 2006;4:e88. http://dx.doi.org/10.1371/journal.pbio.0040088
23. Meng S, Sun Y, Wu X, Tang J, Xu G, Lei Y, et al. Evolutionary dynamics of rabies viruses highlights the importance of China rabies transmission in Asia. Virology. 2011;410:403–9. http://dx.doi.org/10.1016/j.virol.2010.12.011
24. Kissi B, Tordo N, Bourhy H. Genetic polymorphism in the rabies virus nucleoprotein gene. Virology. 1995;209:526–37. http://dx.doi.org/10.1006/viro.1995.1285
25. Mebatsion T, Weiland F, Conzelmann KK. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. J Virol. 1999;73:242–50.
26. Finke S, Mueller-Waldeck R, Conzelmann KK. Rabies virus matrix protein regulates the balance of virus transcription and replication. J Gen Virol. 2003;84:1613–21. http://dx.doi.org/10.1099/vir.0.19128-0
27. Kassis R, Larrous F, Estaquier J, Bourhy H. Lyssavirus matrix protein induces apoptosis by a TRAIL-dependent mechanism involving caspase-8 activation. J Virol. 2004;78:6543–55. http://dx.doi.org/10.1128/JVI.78.12.6543-6555.2004
28. Lentz TL, Wilson PT, Hawrot E, Speicher DW. Amino acid sequence similarity between rabies virus glycoprotein and snake venom curaremimetic neurotoxins. Science. 1984;226:847–8. http://dx.doi.org/10.1126/science.6494916
29. Poch O, Blumberg BM, Bougueleret L, Tordo N. Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. J Gen Virol. 1990;71:1153–62. http://dx.doi.org/10.1099/0022-1317-71-5-1153
30. Kuzmin IV, Wu X, Tordo N, Rupprecht CE. Complete genomes of Aravan, Khujand, Irkut and West Caucasian bat viruses, with special attention to the polymerase gene and non-coding regions. Virus Res. 2008;136:81–90. http://dx.doi.org/10.1016/j.virusres.2008.04.021
31. Zhang S, Zhao J, Liu Y, Fooks AR, Zhang F, Hu R. Characterization of a rabies virus isolate from a ferret badger (*Melogale moschata*) with unique molecular differences in glycoprotein antigenic site III. Virus Res. 2010;149:143–51. http://dx.doi.org/10.1016/j.virusres.2010.01.010
32. Gong W, Jiang Y, Za Y, Zeng Z, Shao M, Fan J, et al. Temporal and spatial dynamics of rabies viruses in China and Southeast Asia. Virus Res. 2010;150:111–8. http://dx.doi.org/10.1016/j.virusres.2010.02.019
33. Saito M, Oshitani H, Orbina JR, Tohma K, de Guzman AS, Kamigaki T, et al. Genetic diversity and geographic distribution of genetically distinct rabies viruses in the Philippines. PLoS Negl Trop Dis. 2013;7:e2144. http://dx.doi.org/10.1371/journal.pntd.0002144
34. Streicker DG, Lemey P, Velasco-Villa A, Rupprecht CE. Rates of viral evolution are linked to host geography in bat rabies. PLoS Pathog. 2012;8:e1002720. http://dx.doi.org/10.1371/journal.ppat.1002720

35. Biek R, Henderson JC, Waller LA, Rupprecht CE, Real LA. A high-resolution genetic signature of demographic and spatial expansion in epizootic rabies virus. Proc Natl Acad Sci U S A. 2007;104:7993–8. http://dx.doi.org/10.1073/pnas.0700741104
36. Faber M, Faber ML, Papaneri A, Bette M, Weihe E, Dietzschold B, et al. A single amino acid change in rabies virus glycoprotein increases virus spread and enhances virus pathogenicity. J Virol. 2005;79:14141–8. http://dx.doi.org/10.1128/JVI.79.22.14141-14148.2005
37. Faber M, Li J, Kean RB, Hooper DC, Alugupalli KR, Dietzschold B. Effective preexposure and postexposure prophylaxis of rabies with a highly attenuated recombinant rabies virus. Proc Natl Acad Sci U S A. 2009;106:11300–5. http://dx.doi.org/10.1073/pnas.0905640106
38. Lafon M. Rabies virus receptors. J Neurovirol. 2005;11:82–7. http://dx.doi.org/10.1080/13550280590900427
39. Tuffereau C, Schmidt K, Langevin C, Lafay F, Dechant G, Koltzenburg M. The rabies virus glycoprotein receptor p75NTR is not essential for rabies virus infection. J Virol. 2007;81:13622–30. http://dx.doi.org/10.1128/JVI.02368-06
40. Kuzmin IV, Shi M, Orciari LA, Yager PA, Velasco-Villa A, Kuzmina NA, et al. Molecular inferences suggest multiple host shifts of rabies viruses from bats to mesocarnivores in Arizona during 2001–2009. PLoS Pathog. 2012;8:e1002786. http://dx.doi.org/10.1371/journal.ppat.1002786

Address for correspondence: Victor Fei Pang, Graduate Institute of Molecular and Comparative Pathobiology, School of Veterinary Medicine, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan, ROC; email: pang@ntu.edu.tw



Persistence and Complex Evolution of Fluoroquinolone-Resistant *Streptococcus pneumoniae* Clone

Debby Ben-David, Mitchell J. Schwaber, Amos Adler, Samira Masarwa, Rotem Edgar, Shiri Navon-Venezia, David Schwartz, Nurith Porat, Tali Kotlovsky, Nikolay Polivkin, Irina Weinberg, Avraham Lazary, Nissim Ohana, and Ron Dagan

Prolonged outbreaks of multidrug-resistant *Streptococcus pneumoniae* in health care facilities are uncommon. We found persistent transmission of a fluroquinolone-resistant *S. pneumoniae* clone during 2006–2011 in a post–acute care facility in Israel, despite mandatory vaccination and fluoroquinolone restriction. Capsular switch and multiple antimicrobial nonsusceptibility mutations occurred within this single clone. The persistent transmission of fluoroquinolone-resistant *S. pneumoniae* during a 5-year period underscores the importance of long-term care facilities as potential reservoirs of multidrug-resistant streptococci.

Institutionalized persons, particularly those >65 years of age, are at high risk for pneumococcal infections (*1*–*3*). The rate of sporadic pneumococcal diseases for nursing home residents is almost 20 times higher than for elderly persons living in the community (*1*).

In September 2008 in Israel, after report of an invasive pneumococcal disease caused by a fluoroquinolone-resistant *Streptococcus pneumoniae* (FQRSP) strain in a patient who had been transferred from a post–acute care facility, an investigation led to discovery that this phenotype had been endemic in the facility for at least 2 years. In the index case-patient, an 81-year-old woman with dementia, bilateral pneumonia and acute respiratory failure developed while she was in a post-acute care facility. Because her condition rapidly deteriorated, she was transferred to a tertiary acute care facility and died within 48 hours. Blood cultures recovered FQRSP. Because fluoroquinolone resistance among *S. pneumoniae* strains is rare in Israel and was infrequently reported in previous pneumococcal outbreaks worldwide (*4*,*5*), we conducted an investigation and attempted to implement measures to limit the spread of the resistant strains. Here we describe prolonged endemicity of a FQRSP clone in the post–acute care facility, its molecular epidemiology, and the effect of infection control measures implemented. The analysis and reporting were approved by the jurisdictional institutional review board of Sourasky Medical Center (Tel Aviv, Israel).

Methods

Setting

The facility is a 307-bed, post–acute care hospital (273 adults, 34 children). Patients are grouped into the following wards: adults and children on long-term mechanical ventilation, rehabilitation, and skilled nursing care. Median duration of hospitalization is 48 days in the rehabilitation wards, 152 days in the skilled nursing wards, and 313 days in the long-term mechanical ventilation wards.

Outbreak Investigation

In September 2008, after report of the patient with FQRSP bloodstream infection, we reviewed the clinical microbiology database at Sourasky Medical Center from January 2006 onward. A clinical case was defined as FQNSP isolated from a clinical specimen from any source. Fifty-two clinical cases of FQRSP were identified, and an outbreak investigation was initiated. However, medical records were found only for 43 patients, and clinical

Author affiliations: National Center for Infection Control, Tel Aviv, Israel (D. Ben-David, M.J. Schwaber, A. Adler, S. Masarwa, R. Edgar, S. Navon-Venezia); Sourasky Medical Center, Tel Aviv (D. Schwartz, N. Porat, T. Kotlovsky, R. Dagan); Ben-Gurion University of the Negev, Beer-Sheva (N. Porat, R. Dagan); and Reuth Medical Center, Tel Aviv (N. Polivkin, I. Weinberg, A. Lazary, N. Ohana)

DOI: http://dx.doi.org/10.3201/eid2005.130142

documentation of physical examination and clinical assessment findings was sparse. We reviewed the medical records for clinical and epidemiologic data, including patients' demographics, underlying diseases, and antimicrobial drug exposure, in the 3 months preceding the isolation.

Interventions

The first intervention, vaccination, began in November 2008. Vaccination with 23-valent pneumococcal polysaccharide vaccine (PPV23) was made mandatory for all patients ≥2 years of age who were admitted to the facility. In addition, all adult patients not previously vaccinated with PPV23 received 1 dose during November and December 2008. Hospitalized and newly admitted unvaccinated children <5 years of age received 1 dose of 7-valent pneumococcal polysaccharide vaccine (PPV7). In addition, children 2–4 years of age received 1 dose of PPV23.

The second intervention, fluoroquinolone restriction, was implemented from January 2010 through October 2011 in all wards. Under the restriction policy, fluoroquinolones were prescribed only after approval by a designated staff physician. Fluoroquinolones were not used for empirical therapy and were approved for definitive therapy only when other therapeutic options were unavailable.

Total fluoroquinolone use since January 2009 as recorded by the central pharmacy was aggregated for each ward into defined daily doses (DDDs) per 1,000 bed-days, as recommended by the World Health Organization (*6*). The study was divided into 3 periods: baseline period (January 2006–January 2009); phase 1: post–vaccination period (February 2009–December 2009); and phase 2: fluoroquinolone-restriction period (January 2010–October 2011).

Point-Prevalence Surveillances

To determine the extent of FQNSP spread among patients and staff members, point-prevalence surveillance was conducted during the baseline period in January 2009. A convenience sample of ≈10 patients from each of the 9 wards and 20 staff members from these wards were selected for screening. Oropharyngeal and nasopharyngeal swab samples were taken from all persons by using the Transwab Pernasal Amies Plain wire swabs (Medical Wire, Corsham, UK). Endotracheal aspirates were obtained by using a suction catheter introduced through tracheostomy tubes.

To evaluate the effects of vaccination and fluoroquinolone restriction on FQNSP, we conducted follow-up point-prevalence surveillances during December 2009–January 2010 and May–June 2011. In the second and third surveys we increased the sample size, screening all patients hospitalized in a convenience sample of 3 wards in addition to a sample of 10 patients from each of the other wards. These wards represented all types of wards in the facility.

Microbiological Methods

Pneumococcal Isolation, Identification, and Susceptibility Testing

Specimens were transported to the clinical laboratory at Sourasky Medical Center. Specimens were streaked onto either tryptic soy agar with 5% sheep blood and gentamicin (5 mg/L) (first and second surveys) or Streptococcal Select Agar plates (Hy-labs, Rehovot, Israel) (third survey) and incubated overnight at 37°C in 5% CO_2; the 2 methods were validated in our laboratory. The selective plates were compared with tryptic soy agar–5% sheep blood and had similar ability to support the growth of *S. pneumoniae* (data not shown). Pneumococcal identification and antimicrobial susceptibility testing were performed with the VITEK-2 system by using the GP and AST-GP68 cards, respectively (bioMérieux, Marcy l'Etoile, France) according to Clinical and Laboratory Standards Institute guidelines (*7*). MICs of ofloxacin, levofloxacin, and moxifloxacin also were tested in representative isolates by Etest (AB Biodisk, Solna, Sweden). Pneumococci were defined as resistant to ofloxacin if the ofloxacin MIC was ≥8 μg/mL (FQRSP). Fluoroquinolone intermediately resistant *S. pneumoniae* (FQISP) was defined as MIC = 4 μg/mL. Penicillin-nonsusceptible strains were defined as those with penicillin MIC ≥2 μg/mL.

Clonal Analysis

Serogrouping and serotyping were performed by the quellung reaction using antiserum provided by Statens Serum Institute (Copenhagen, Denmark) (*8*). We determined the genetic relatedness of *S. pneumoniae* strains by pulsed-field gel electrophoresis (PFGE) analysis, as described (*9*). Selected isolates representing all PFGE clusters were characterized by multilocus sequence typing (MLST) as described by Enright and Spratt (*10*). The sequences (alleles) at each locus were compared with those at the MLST website (www.mlst.net), and sequence types (STs) were assigned.

Mechanisms of Fluoroquinolone Resistance

Mutations in the quinolone resistance–determining regions (QRDR) of genes encoding subunits of topoisomerase IV and DNA gyrase were assessed. Representatives from each serotype in PFGE and resistance level were tested. Primers amplifying the QRDR (*11*) were designed for each of the 4 genes: *parC* primers, F-CAAAACATGTCCCTGGAGGA and R-GCAGCATCTATGACCTCAGC; *parE* primers, F-TCAAGTCTGCCATTACCAAGG and R-ACCCGCACCAATGGTATAAA; *gyrA* primers, F2-GACAAAGGAGATGAAGGCAAG and R2-GAAAATCTGGTCCAGGCAAG; *gyrB* primers, F-GGGAAATAGCGAAGTGGTCA and R-GTACGAATGTGGGCTCCAT. PCR on lysates with primers as above

using Hot Star Taq (QIAGEN, Hilden, Germany) was performed as follows: 95°C for 15 min and 39 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, followed by an extension step of 72°C for 10 min, and the products were sequenced (HyLab, Rehovot, Israel). Sequences were analyzed by BLAST (http://blast.ncbi.nlm.nih.gov) against 1 of the 2 identical sequenced pneumococcal strains in the database (NC_008533 *Streptococcus pneumoniae* D39 and AE007317).

Statistical Analysis

The effect of the intervention was assessed during the 3 periods: baseline period, phase 1 (February 2009–December 2009), and phase 2 (January 2010–October 2011). We assumed a lag time of 2–4 weeks for demonstrating vaccine efficacy and therefore defined the postvaccination period as beginning ≈1 month after vaccination. We used segmented Poisson regression analysis of interrupted time series to compare FQRSP incidence during the 3 periods. FQRSP incidence was measured as cases per 10,000 patient-days. A p value ≤0.05 was considered significant.

Results

Clinical Cases of FQRSP during the Baseline Period

Before November 2008, pneumococcal vaccine was not administered at admission to the facility. Only 40 (13%) of 310 patients had received pneumococcal vaccine before hospitalization. During January 2006–December 2008, *S. pneumoniae* was isolated from 66 patients. Of these, 52 (79%) isolates were fluoroquinolone-resistant, 11 (17%) were fluoroquinolone-susceptible, and 3 (5%) were intermediate. FQRSP was isolated predominantly from sputum (51 of 52 isolates). Most (45 [87%] of 52) FQRSP isolates were nonsusceptible to penicillin. Resistance to erythromycin, tetracycline, and trimethoprim/sulfamethoxazole was found in 8%, 6%, and 13% of isolates, respectively. In contrast to the high FQRSP isolation rate in the facility, the same laboratory detected fluoroquinolone resistance in only 4 (2%) of 240 of pneumococcal isolates from sputum cultures from patients in a tertiary acute care facility during that period.

Medical records were available for 43 patients with FQRSP. Their median age was 59 years (range 20–94 years). Twenty-nine (67%) patients were male. The median length of stay at the facility before FQRSP isolation was 329 days (range 3 days–26 years). Antimicrobial drug use was high in this population: 51% of patients had received ≥1 antimicrobial agents in the 3 months preceding isolation. Only 5 (12%) of the 43 patients received fluoroquinolones in the 3 months preceding isolation. Symptoms associated with detection of FQRSP included fever (23 [53%] patients) and respiratory deterioration (27 [63%]).

Baseline Point-Prevalence Surveillance: Serotyping and Clonal Analysis

The baseline survey comprised 84 (93%) of the 90 eligible patients and 20 (4%) of 525 health care workers. Asymptomatic colonization with *S. pneumoniae* was detected among 20 patients (16 [23%] of 69 adults; 4 [27%] of 15 children) and 1 (5%) health care worker. Of the colonized patients, 12 (60%) had FQNSP. This represented 14% of the sampled patients: 10 (14%) of 69 adults, all with FQRSP; and 2 (13%) of 15 children, all with FQISP. The isolate from the health care worker was fluoroquinolone susceptible.

The FQRSP isolates belonged to 2 different serotypes and 3 different PFGE types (Figure 1). All belonged to a single ST (ST156). The 3 FQISP isolates from children belonged to serotype 19F. All belonged to a single clone that was different from the adult clone.

Mechanism of Resistance

We sequenced 7 isolates representing each PFGE type and resistance profile (Table 1, Appendix, wwwnc.cdc.gov/EID/article/20/5/13-0142-T1.htm). Four isolates from adults (nos. 109, 182, 129, 116) had identical mutations previously reported in quinolone resistance: S81F in *gyrA* and S79Y in *parC*. In addition, we observed 3 silent mutations in *gyrB* and 1 silent mutation in *parC*. The silent mutation in *parC* was common to all isolates, including those from the 2 children and the fluoroquinolone-susceptible reference isolate. The 2 isolates from children (nos. 177, 190) had mutations in *parE*; 1 silent mutation and an additional I460V mutation. Isolate no. 200, a susceptible reference, also had an I460V mutation. This mutation is not reported to yield fluoroquinolone resistance. Isolate no. 177, which had a higher degree of resistance to ciprofloxacin and levofloxacin than did isolate no. 190, had 2 additional mutations, E474K and A326V.

Interventions

A total of 197 (83%) eligible patients received PPV23; the remaining 41 patients refused. Seventeen (93%) eligible patients received PCV7.

During 2009, the mean fluoroquinolone DDD in the facility was 36.1. After implementing fluoroquinolone restriction, the mean DDD decreased to 16.7 during 2010 but then increased again to 29.3 during 2011. Total antimicrobial drug use did not change during the entire follow up (DDD was 196, 182, and 208 during 2009, 2010, and 2011, respectively).

Effect of the Interventions

During the baseline period, the rate of new clinical cases decreased (–0.1021) (Table 2, Figure 2). After implementation of mandatory vaccination, an additional decrease in incidence was observed (–0.4675). However,

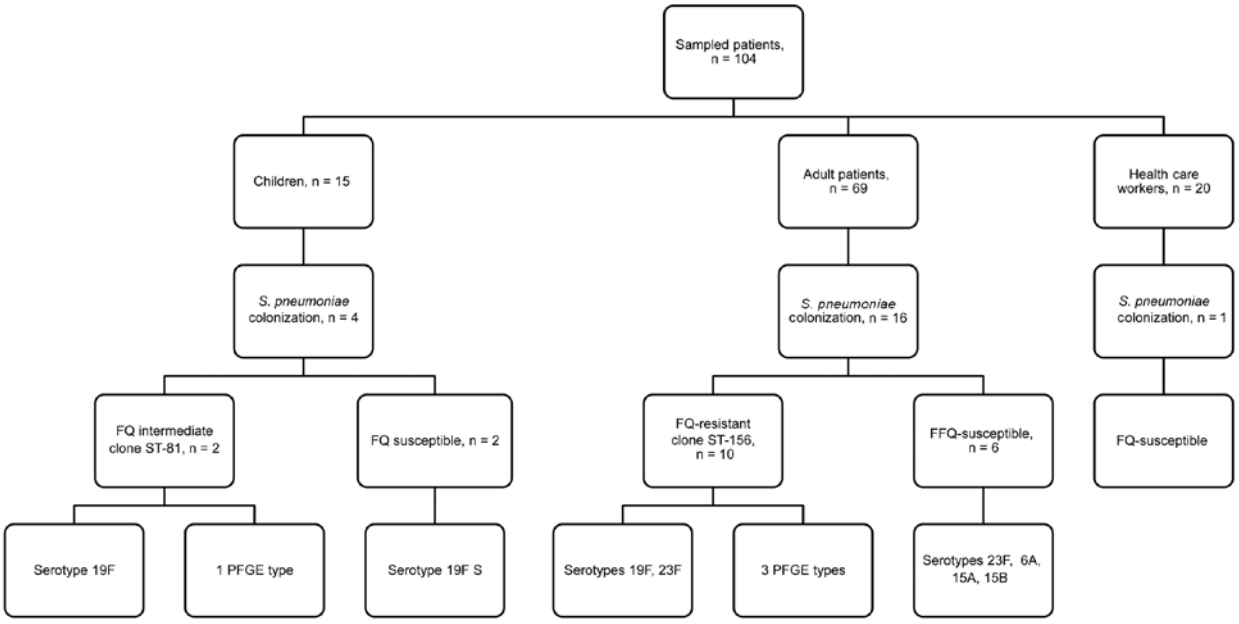


Figure 1. Serotyping and clonal analysis of the first point-prevalence survey of *Streptococcus pneumoniae* infection in a post–acute care facility, Israel, 2006–2011. FQ fluoroquinolone; ST, sequence type; FQISP, fluoroquinolone-intermediate *S. pneumoniae*; FQRSP fluoroquinolone-resistant *S. pneumoniae*.

after the restriction began on use of antimicrobial drugs, incidence again increased (0.5489).

In the second and third surveys, 154 (90%) of 172 and 165 (97%) of 171 eligible patients were included, respectively. The prevalence of FQRSP decreased from the initial survey to the second survey. Prevalence increased in the third survey (Table 3).

Discussion

The ability of *S. pneumoniae* to cause outbreaks in long-term care facilities has been reported (*2*,*12*,*13*). Most reports have described invasive infections over a few weeks that involved on average 10–20 patients (*2*,*12*). Also well documented is the ability of multiresistance serotypes to spread internationally and to become predominant clones in multiple geographic areas (*14*–*16*). However, outbreaks of FQRSP have rarely been reported (*4*,*5*). This study describes prolonged transmission of FQRSP in a post–acute care hospital during at least 5 years despite implementation of mandatory vaccination and fluoroquinolone restriction. The prolonged endemicity demonstrates the potential for FQRSP strains to persist within an institution for several years; undergo capsular switch; and in the process, acquire new resistance mutations to multiple antimicrobial drugs.

Table 2. Trends in the incidence of fluoroquinolone-resistant *Streptococcus pneumonia*e in a post–acute care facility, Israel, 2006–2011

Period	Trend coefficient (SE)	p value
Preintervention	–0.1021 (0.0348)	0.0065
First intervention	–0.4675 (0.1969)	0.0208
Second intervention	0.5489 (0.2071)	0.0102

Most health care–associated *S. pneumoniae* infections are reported from long-term care facilities, and residence in a long-term care facility is an independent risk factor (*2*,*3*,*17*,*18*). In the study reported here, despite the persistence of FQRSP in the facility, we did not notice spread of FQRSP to other settings or health care facilities. During the 5-year follow-up, no FQRSP outbreaks were reported to the National Center for Infection Control from elsewhere in the country, suggesting a high fitness cost of the fluoroquinolone resistance. Despite the prolonged presence of FQRSP clones in the facility, most colonization did not progress to invasive disease. Indeed, the dominant serotypes, 19F and 23F, are not typically associated with invasive disease in adults (*19*). However, reduced virulence as a result of antimicrobial resistance cannot be ruled out. It was previously suggested that penicillin resistance is associated with decreased virulence (*14*,*15*,*20*). However, to the best of our knowledge, no clinical evidence of reduced virulence in FQRSP is available.

Despite the recommendation for PPV23 administration to persons at high risk for pneumococcal pneumonia (*21*), longstanding controversy exists over its efficacy in preventing noninvasive disease (*22*). A recent randomized controlled study demonstrated PPV23 efficacy in preventing pneumococcal pneumonia and reducing associated death in nursing home residents (*23*). Although most successful interventions in long-term care facilities included use of vaccinations (*12*,*24*,*25*), evidence is limited for

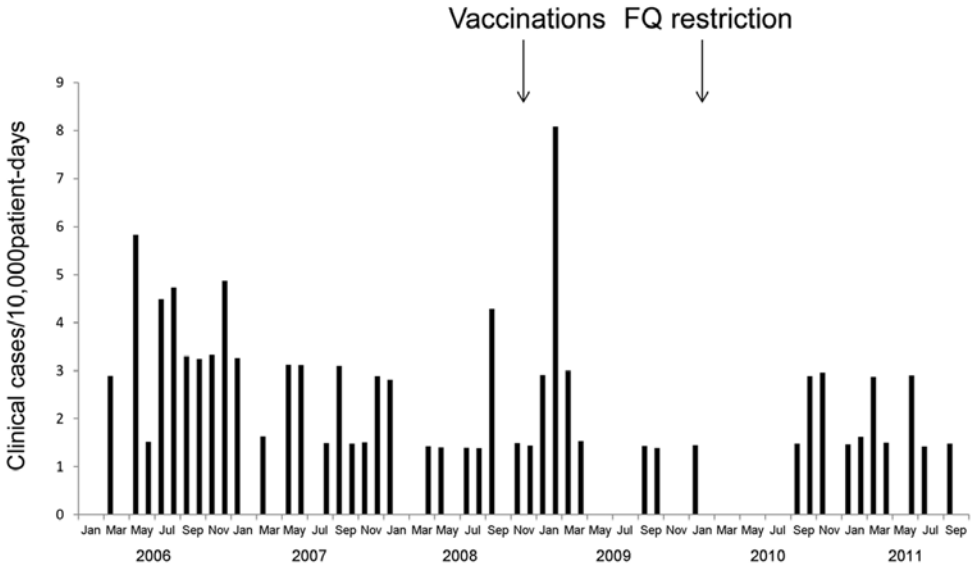


Figure 2. Clinical isolates of FQ-resistant *Streptococcus pneumoniae* in a post–acute care facility, Israel, 2006–2011. FQ, fluoroquinolone.

effectiveness of PPV23 against pneumococcal pneumonia or nasopharyngeal colonization. Furthermore, PPV23 is not thought to reduce carriage and thus cannot be an effective tool to reduce transmission. Preliminary studies suggest that pneumococcal conjugate vaccines induce higher levels of immunity among adults than does PPV23 (*26*). However, further studies are needed to assess whether pneumococcal conjugate vaccines will be more effective in pneumonia prevention among the elderly.

In a recent review, interventions implemented in 28 cluster reports included vaccination, chemoprophylaxis, and infection control measures (*27*). Most studies have reported successful interventions. However, in most cases, outbreak duration and follow-up both were short (median 3 months). In the current study, after implementing mandatory vaccination, we observed an initial decrease in the incidence of FQRSP. However, a decreasing trend was demonstrated even before the intervention. During the prolonged follow-up, we noticed continuous spread of the resistant clones in the facility. Chemoprophylaxis was used in most reported interventions (*27*). However, this strategy might be associated with selection of new resistant strains or mechanisms. Specifically, FQRSP was detected in a long-term care facility, after a short chemoprophylaxis course with combination therapy (*5*). Furthermore, in the present outbreak, because of diversity of the antimicrobial resistance and multiresistance phenotypes, any chosen antimicrobial drug would have further selected and promoted the already prevalent resistant strains, explaining its prolonged persistence. Restriction of fluoroquinolones did not result in a sustained decrease in the incidence of FQRSP. Prior fluoroquinolone treatment was not common in the study population reported here, but the overall use of antimicrobial drugs was not reduced in the studied facility even after the interventions began. We are assessing the effect of antimicrobial drug stewardship and improved compliance with standard precautions on the control of the spread of FQRSP in the facility.

The current FQRSP clone (ST156) comprised 2 different serotypes, which suggests that capsular switch occurred somewhere after introduction of the clone to the

Table 3. Results of 3 point-prevalence surveys about *Streptococcus pneumoniae* conducted among residents in a post–acute care facility, Israel, 2006–2011*

Survey and age group, y	No. patients screened	FQRSP		FQISP		FQ-susceptible *S. pneumoniae*	
		No. (%)	Serotype (no. isolates)	No. (%)	Serotype (no. isolates)	No. (%)	Serotype (no. isolates)
First							
<18	15	0	–	2 (13)	15A (1), 19F (1)	2 (13)	19F (2)
>18	69	10 (15)	19F (6), 23F (4)	0	_	6 (8)	15A (1), 15B (2), 23F(1), 6A (2)
Second							
<18	24	0	–	0	_	1 (4)	15A (1)
>18	130	12 (9)	23F (8), 19F (3); negative (1)	0	_	2 (1)	17F (1), 6A (1)
Third							
<18	25	0	–	2 (8)	17F (1), 19F (1)	4 (16)	15A (3), 17F (1)
>18	140	19 (13)	23F (12), 19F (7)	0	_	5 (3.6)	17F (4), negative (1)

*FQRSP, fluoroquinolone-resistant *S. pneumoniae*; FQISP, fluoroquinolone intermediate *S. pneumoniae*; FQ, fluoroquinolone; –, no isolates.

facility. The capacity of pneumococci for transformation of capsular type has been described in several studies (*28*,*29*), but in the current study, the location of the event can be assumed with a high degree of certainty. Capsular switch events have been defined as 2 isolates identified by MLST as being closely related but expressing different serotypes. Acquisition of a new capsule may have provided an advantage against the host immune system.

During the past few years, FQRSP has been reported from several countries, although the prevalence remains low (*16*,*30*,*31*). Fluoroquinolone nonsusceptibility among pneumococci results mainly from point mutations in the QRDR topoisomerase genes (*32*). In the current study, identical mutations were found in different serotypes among the strains in adults. This finding suggests that the mutation occurred before capsular switch. Different mutations occurred among the children's strains and were associated with intermediate resistance. The difference between children and adults might be due to low rates of fluoroquinolone use among children and the relative separation of the children from adults in the family.

Our study has several limitations. First, because microbiological data were not available before January 2006, we cannot determine when the resistant clone was introduced into the facility. Second, clinical isolates for 2006–2008 were not available for analysis. Therefore, we performed molecular characterization only for strains found in the point-prevalence survey. Consequently, we cannot determine the dynamics of resistance development in the facility. Third, we conducted point-prevalence surveys among a sample of hospitalized patients and not among all hospitalized patients. Because we used a convenience sample, selection bias, although unlikely, cannot be categorically excluded. We assessed the effect of both vaccination and fluoroquinolone restriction. However, because the second phase comprised 2 ongoing interventions, we cannot assess separately the effect of each intervention. Finally, we did not conduct a case–control study and therefore risk factors for FQRSP acquisition cannot be assessed.

The persistent transmission of FQRSP during a 5-year period underscores the importance of long-term care facilities as potential reservoir of multidrug resistant particularly FQRSP. Further work is needed to identify optimal strategies to prevent the emergence and spread of resistant pneumococcal strains in long-term care facilities, including potential use of pneumococcal conjugate vaccines, antimicrobial stewardship, and infection control interventions to interrupt transmission.

Dr Ben-David is a hospital epidemiologist at the National Center for Infection Control in Israel. Her research emphasis is multidrug-resistant pathogens.

References

1. Maruyama T, Niederman MS, Kobayashi T, Kobayashi H, Takagi T, D'Alessandro-Gabazza CN, et al. A prospective comparison of nursing home–acquired pneumonia with hospital-acquired pneumonia in non-intubated elderly. Respir Med. 2008;102:1287–95. http://dx.doi.org/10.1016/j.rmed.2008.03.027
2. Ihekweazu C, Basarab M, Wilson D, Oliver I, Dance D, George R, et al. Outbreaks of serious pneumococcal disease in closed settings in the post-antibiotic era: a systematic review. J Infect. 2010;61:21–7. http://dx.doi.org/10.1016/j.jinf.2010.03.032
3. Kupronis BA, Richards CL, Whitney CG. Invasive pneumococcal disease in older adults residing in long-term care facilities and in the community. J Am Geriatr Soc. 2003;51:1520–5. http://dx.doi.org/10.1046/j.1532-5415.2003.51501.x
4. Weiss K, Restieri C, Gauthier R, Laverdiere M, McGeer A, Davidson RJ, et al. A nosocomial outbreak of fluoroquinolone-resistant *Streptococcus pneumoniae.* Clin Infect Dis. 2001;33:517–22. http://dx.doi.org/10.1086/322658
5. Carter RJ, Sorenson G, Heffernan R, Kiehlbauch JA, Kornblum JS, Leggiadro RJ, et al. Failure to control an outbreak of multidrug-resistant *Streptococcus pneumoniae* in a long-term–care facility: emergence and ongoing transmission of a fluoroquinolone-resistant strain. Infect Control Hosp Epidemiol. 2005;26:248–55. http://dx.doi.org/10.1086/502534
6. WHO Collaborating Centere for Drug Statisics Methodology. Guidelines for ATC classification and DDDs assignment. Oslo, Noway: The Centere; 2002.
7. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twenty-first informational supplement. Approved standard MS100–S20. Wayne (PA): The Institute; 2006.
8. Austrian R. The quellung reaction, a neglected microbiologic technique. Mt Sinai J Med. 1976;43:699–709.
9. Porat N, Arguedas A, Spratt BG, Trefler R, Brilla E, Loaiza C, et al. Emergence of penicillin-nonsusceptible *Streptococcus pneumoniae* clones expressing serotypes not present in the antipneumococcal conjugate vaccine. J Infect Dis. 2004;190:2154–61. http://dx.doi.org/10.1086/425908
10. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology. 1998;144:3049–60. http://dx.doi.org/10.1099/00221287-144-11-3049
11. Maeda Y, Murayama M, Goldsmith CE, Coulter WA, Mason C, Millar BC, et al. Molecular characterization and phylogenetic analysis of quinolone resistance-determining regions (QRDRs) of *gyrA, gyrB, parC* and *parE* gene loci in viridans group streptococci isolated from adult patients with cystic fibrosis. J Antimicrob Chemother. 2011;66:476–86. http://dx.doi.org/10.1093/jac/dkq485
12. Nuorti JP, Butler JC, Crutcher JM, Guevara R, Welch D, Holder P, et al. An outbreak of multidrug-resistant pneumococcal pneumonia and bacteremia among unvaccinated nursing home residents. N Engl J Med. 1998;338:1861–8. http://dx.doi.org/10.1056/NEJM199806253382601
13. Tan CG, Ostrawski S, Bresnitz EA. A preventable outbreak of pneumococcal pneumonia among unvaccinated nursing home residents in New Jersey during 2001. Infect Control Hosp Epidemiol. 2003;24:848–52. http://dx.doi.org/10.1086/502148
14. Winston LG, Perlman JL, Rose DA, Gerberding JL. Penicillin-nonsusceptible *Streptococcus pneumoniae* at San Francisco General Hospital. Clin Infect Dis. 1999;29:580–5. http://dx.doi.org/10.1086/598637
15. Yu VL, Chiou CC, Feldman C, Ortqvist A, Rello J, Morris AJ, et al. An international prospective study of pneumococcal bacteremia: correlation with in vitro resistance, antibiotics administered, and clinical outcome. Clin Infect Dis. 2003;37:230–7. http://dx.doi.org/10.1086/377534

16. Powis J, McGeer A, Green K, Vanderkooi O, Weiss K, Zhanel G, et al. In vitro antimicrobial susceptibilities of *Streptococcus pneumoniae* clinical isolates obtained in Canada in 2002. Antimicrob Agents Chemother. 2004;48:3305–11. http://dx.doi.org/10.1128/AAC.48.9.3305-3311.2004
17. Ho PL, Tse WS, Tsang KW, Kwok TK, Ng TK, Cheng VC, et al. Risk factors for acquisition of levofloxacin-resistant *Streptococcus pneumoniae*: a case–control study. Clin Infect Dis. 2001;32:701–7. http://dx.doi.org/10.1086/319222
18. Vanderkooi OG, Low DE, Green K, Powis JE, McGeer A. Predicting antimicrobial resistance in invasive pneumococcal infections. Clin Infect Dis. 2005;40:1288–97. http://dx.doi.org/10.1086/ 429242
19. Sá-Leão R, Pinto F, Aguiar S, Nunes S, Carriço JA, Frazão N, et al. Analysis of invasiveness of pneumococcal serotypes and clones circulating in Portugal before widespread use of conjugate vaccines reveals heterogeneous behavior of clones expressing the same serotype. J Clin Microbiol. 2011;49:1369–75. http://dx.doi.org/10.1128/JCM.01763-10
20. Rieux V, Carbon C, Azoulay-Dupuis E. Complex relationship between acquisition of β-lactam resistance and loss of virulence in *Streptococcus pneumoniae.* J Infect Dis. 2001;184:66–72. http://dx.doi.org/10.1086/320992
21. Centers for Disease Control and Prevention. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 1997;46 (RR-8):1–24.
22. Huss A, Scott P, Stuck AE, Trotter C, Egger M. Efficacy of pneumococcal vaccination in adults: a meta-analysis. CMAJ. 2009;180:48–58. http://dx.doi.org/10.1503/cmaj.080734
23. Maruyama T, Taguchi O, Niederman MS, Morser J, Kobayashi H, Kobayashi T, et al. Efficacy of 23-valent pneumococcal vaccine in preventing pneumonia and improving survival in nursing home residents: double blind, randomised and placebo controlled trial. BMJ. 2010;340:c1004. http://dx.doi.org/10.1136/bmj.c1004
24. Centers for Disease Control and Prevention. Outbreaks of pneumococcal pneumonia among unvaccinated residents of chronic-care facilities—Massachusetts, October 1995, Oklahoma, February, 1996, and Maryland, May–June 1996. MMWR Morb Mortal Wkly Rep. 1997;46:60–2.
25. Sheppard DC, Bartlett KA, Lampiris HW. *Streptococcus pneumoniae* transmission in chronic-care facilities: description of an outbreak and review of management strategies. Infect Control Hosp Epidemiol. 1998;19:851–3. http://dx.doi.org/10.2307/30141564
26. Goldblatt D, Southern J, Andrews N, Ashton L, Burbidge P, Woodgate S, et al. The immunogenicity of 7-valent pneumococcal conjugate vaccine versus 23-valent polysaccharide vaccine in adults aged 50–80 years. Clin Infect Dis. 2009;49:1318–25. http://dx.doi.org/10.1086/606046
27. Basarab M, Ihekweazu C, George R, Pebody R. Effective management in clusters of pneumococcal disease: a systematic review. Lancet Infect Dis. 2011;11:119–30. http://dx.doi.org/10.1016/S1473-3099(10)70281-4
28. Barnes DM, Whittier S, Gilligan PH, Soares S, Tomasz A, Henderson FW. Transmission of multidrug-resistant serotype 23F *Streptococcus pneumoniae* in group day care: evidence suggesting capsular transformation of the resistant strain in vivo. J Infect Dis. 1995;171:890–6. http://dx.doi.org/10.1093/infdis/171.4.890
29. Nesin M, Ramirez M, Tomasz A. Capsular transformation of a multidrug-resistant *Streptococcus pneumoniae* in vivo. J Infect Dis. 1998;177:707–13. http://dx.doi.org/10.1086/514242
30. Rodriguez-Avial I, Ramos B, Rios E, Cercenado E, Ordobas M, Sanz JC. Clonal spread of levofloxacin-resistant streptococcus pneumoniae invasive isolates in Madrid, Spain, 2007 to 2009. Antimicrob Agents Chemother. 2011;55:2469–71. http://dx.doi.org/10.1128/AAC.01380-10
31. Karlowsky JA, Jones ME, Draghi DC, Thornsberry C, Sahm DF, Volturo GA. Prevalence and antimicrobial susceptibilities of bacteria isolated from blood cultures of hospitalized patients in the United States in 2002. Ann Clin Microbiol Antimicrob. 2004;3:7. http://dx.doi.org/10.1186/1476-0711-3-7
32. Richter SS, Heilmann KP, Beekmann SE, Miller NJ, Rice CL, Doern GV. The molecular epidemiology of *Streptococcus pneumoniae* with quinolone resistance mutations. Clin Infect Dis. 2005;40:225–35. http://dx.doi.org/10.1086/426817

Address for correspondence: Debby Ben-David, National Center for Infection Control, Tel Aviv Sourasky Medical Center, 6 Weizmann St, Tel Aviv 64239, Israel; email: debby.bendavid@sheba.health.gov.il

Carriage Rate and Effects of Vaccination after Outbreaks of Serogroup C Meningococcal Disease, Brazil, 2010

Marco Aurelio Palazzi Sáfadi, Telma Regina Marques Pinto Carvalhanas, Ana Paula de Lemos, Maria Cecilia Outeiro Gorla, Maristela Salgado, Lucila O. Fukasawa, Maria Gisele Gonçalves, Fabio Higa, Maria Cristina Cunto Brandileone, Claudio Tavares Sacchi, Ana Freitas Ribeiro, Helena Keico Sato, Lucia Ferro Bricks, and José Cassio de Moraes

During 2010, outbreaks of serogroup C meningococcal (MenC) disease occurred in 2 oil refineries in São Paulo State, Brazil, leading to mass vaccination of employees at 1 refinery with a meningococcal polysaccharide A/C vaccine. A cross-sectional study was conducted to assess the prevalence of meningococci carriage among workers at both refineries and to investigate the effect of vaccination on and the risk factors for pharyngeal carriage of meningococci. Among the vaccinated and nonvaccinated workers, rates of overall meningococci carriage (21.4% and 21.6%, respectively) and of MenC carriage (6.3% and 4.9%, respectively) were similar. However, a MenC strain belonging to the sequence type103 complex predominated and was responsible for the increased incidence of meningococcal disease in Brazil. A low education level was associated with higher risk of meningococci carriage. Polysaccharide vaccination did not affect carriage or interrupt transmission of the epidemic strain. These findings will help inform future vaccination strategies.

In Brazil, meningococcal disease is endemic; 1.5–2.0 cases per 100,000 inhabitants were reported during 2000–2009. Since 2002, a substantial increase has been observed in the proportion of cases attributed to meningococcus serogroup C (MenC) that is associated with the sequence type (ST) 103 complex, and MenC is currently responsible for most cases of meningococcal disease in Brazil (*1*–*3*).

Author affiliations: Faculdade de Ciências Médicas da Santa Casa, São Paulo, Brazil (M.A.P. Sáfadi, J.C. de Moraes); Centro de Vigilância Epidemiológica Alexandre Vranjac, São Paulo (T.R.M.P. Carvalhanas, A.F. Ribeiro, H.K. Sato); Instituto Adolfo Lutz, São Paulo (A.P. de Lemos, M.C.O. Gorla, M. Salgado, M.G. Gonçalves, L.O. Fukasawa, F. Higa, M.C.C. Brandileone, C.T. Sacchi); and Sanofi Pasteur Vaccines, São Paulo (L.F. Bricks)

DOI: http://dx.doi.org/10.3201/eid2005.130948

Several outbreaks of MenC disease have been reported in Brazil in recent years (*2*,*4*–*6*). To control these outbreaks, chemoprophylaxis is administered to contacts of infected persons, and vaccination is often recommended for persons in age groups at higher risk for infection. In these reactive vaccination campaigns, meningococcal C conjugate (MCC) vaccine use is restricted to children <2 years of age because of cost and supply issues; meningococcal A/C polysaccharide vaccine is recommended for persons ≥2 years of age (*1*–*3*).

Published data describing meningococci carriage in Brazil are limited. Few studies have been conducted that assess 1) the role of carriage prevalence in the dynamics of carriage and disease or 2) the potential effect of control programs, such as vaccination, on the transmission of meningococci. Thus, we conducted a cross-sectional study with the primary objective of assessing the prevalence of meningococcal carriage among workers at 2 oil refineries in São Paulo State, Brazil, where outbreaks of MenC disease occurred in 2010. We also investigated the effect of meningococcal A/C polysaccharide vaccination and risk factors on pharyngeal carriage of meningococci.

Methods

During March 29–June 30, 2010, an outbreak of MenC disease, associated with the ST103 complex, occurred in an oil refinery (Refinery A) with 17,590 workers in São Paulo State, Brazil. A total of 18 cases and 3 deaths (case-fatality rate 16.7%) were associated with the outbreak. Six of the cases and 2 deaths involved Refinery A workers, and 12 of the cases and 1 death involved contacts (family members) of the refinery workers. The case-patients were residents of Cosmópolis, a municipality with 59,000 inhabitants located near Refinery A.

On March 29, health authorities were notified of the first 3 case-patients (2 adult workers at Refinery A and an 8-month-old child whose father worked at Refinery A). An investigation was initiated, and chemoprophylaxis with rifampin was recommended for all close contacts of the 3 index case-patients. During the following 2 weeks, 5 new cases of MenC disease were identified (3 in Refinery A workers and 2 in children who were relatives of Refinery A workers). With these new cases, the incidence of meningococcal disease reached 34.1 cases/100,000 persons at Refinery A. Meningococcal A/C polysaccharide vaccination was recommended for all 17,590 workers at Refinery A. Vaccination began on April 16, and 1 week later, 91% coverage of workers at Refinery A was achieved. However, despite the vaccination program, 10 new cases of MenC disease occurred: 9 cases were in family contacts and 1 case was in a Refinery A worker who had received vaccine 1 day before symptom onset.

The incidence of MenC disease in Cosmópolis subsequently reached 20.2 cases/100,000 persons. Cases occurred in relatives (8 months to 16 years of age) of Refinery A workers, prompting a mass vaccination of 18,571 inhabitants of Cosmópolis who were 2 months to 19 years of age. Vaccination began on June 30, and 90.5% coverage was achieved 1 week later. Infants and toddlers received MCC vaccine, and persons 2–19 years of age received meningococcal A/C polysaccharide vaccine. In the months following the vaccination campaign, no more MenC cases were reported, and the outbreak was considered controlled.

The second outbreak of MenC disease occurred in a refinery with 16,000 workers (Refinery B) in São José dos Campos, a city with 610,095 inhabitants in São Paulo State. On July 10, 2010, a worker at Refinery B was reported to have MenC disease, and on July 18, a second worker was reported to be infected. An investigation identified 10 other reported cases in São José dos Campos during April–July, 2010; the 10 cases were in children <4 years of age who were household contacts of Refinery B workers. Of the 12 identified case-patients, 6 died. As in Cosmópolis, these initial cases were considered the index cases. In Refinery B, the incidence of meningococcal disease reached 12.5 cases/100,000 persons, and a decision was made to provide chemoprophylaxis, but not vaccine, to all close contacts of index case-patients. On August 8, 1 new case of meningococcal disease was reported in a family contact of a Refinery B worker; no further cases were reported in 2010.

Beginning in December 2010, we conducted a cross-sectional study of 483 workers (18–39 years of age) from Refinery A, where mass vaccination had been recommended, and Refinery B, where mass vaccination had not been advised. All study participants gave informed consent. A questionnaire was used to obtain information regarding age, sex, recent respiratory tract infections, active and passive smoking, alcohol consumption, recent antimicrobial drug use, length of employment at the refinery, number of household members living in the same room, level of education, and meningococcal A/C polysaccharide vaccination status.

Specimen Collection

During the first 2 weeks of December, 2010, we obtained oropharyngeal swab samples from 483 refinery workers (238 vaccinated workers from Refinery A and 245 nonvaccinated workers from Refinery B). The samples were immediately put into transport medium (*7*) and, within 4–5 h, sent to the Adolfo Lutz Institute (São Paulo, Brazil), the National Reference Laboratory for Bacterial Meningitis, where they were stored until use. The stored oropharyngeal swabs were plated onto selective medium, and after 24–48 h of incubation at 37°C (±2°) in 5% CO_2, the samples were inspected. Samples with meningococcus-like colonies were subcultured on blood agar medium for species identification. Isolates identified as *Neisseria meningitidis* were serogrouped by using an agglutination test. Antisera were obtained for serogroups A, B, C, E, W, X, Y, and Z (*8*,*9*).

DNA Extraction and Real-Time PCR

DNA from each sample was extracted and purified by using the QIAamp DNA MiniKit (QIAGEN, Alameda, CA, USA) or a similar testing kit according to the manufacturer's instructions. Primers and fluorescent probes were used for the detection of *N. meningitidis ctrA* (*10*) and *sodC* genes by real-time PCR (*11*). Samples positive for *N. meningitidis* were genotyped by using primers and fluorescent probes for *N. meningitidis* serogroups A, B, C, W, and X.

Serotyping and Multilocus Sequence Typing

Serotyping for all *N. meningitidis* isolates was performed by dot blot analysis, using whole-cell suspensions as described (*12*). Multilocus sequence typing was performed according to the methods of Maiden et al. (*13*). Primers, determination of sequence alleles, and designation of sequence types are described on the Neisseria Multi Locus Sequence Typing website (http://neisseria.org/nm/typing/mlst).

Statistical Analyses

Using an estimate that the prevalence of meningococci carriage among adults would be ≈18% (±5%), we calculated that ≈225 study participants from each refinery would be needed to analyze all variables. Demographic data for all participants and typing results of *N. meningitidis* isolates were entered into an EpiInfo database (wwwn.cdc.gov/epiinfo/) and compared by using the 2-sided Fisher exact test. Assessment of risk factors was performed using Fisher exact test.

Results

Of the 483 oropharyngeal samples tested, 104 (21.5%; 95% CI 18.0%–25.5%) were positive for meningococci. Carriage rates were similar among workers from both refineries (21.4% vs. 21.6%). Of the 104 positive samples, 95 were detected by culture and real-time PCR, 1 was detected by culture only, and 8 were detected by real-time PCR only.

The serogroup and genogoup could be determined for 56 of the 104 meningococci-positive samples: 27 (48.2%) were serogroup C, 9 (16.1%) serogroup B, 8 (14.3%) serogroup E, 7 (12.5%) serogroup Y, and 5 (8.9%) serogroup W. The serogroup could not be determined for 48 (46.1%) isolates. The difference in MenC carriage rates among workers at the 2 refineries was not significant: 6.3% at Refinery A and 4.9% at Refinery B (p = 0.48) (Table 1).

Serotyping and Multilocus Sequence Typing

A total of 38 different serotype–serosubtype antigen combinations were identified among the 96 *N. meningitidis* isolates. Among MenC isolates, phenotype C:23:P.14–6 was the most prevalent (10/13 [77%]). Eleven different STs were found among 27 isolates characterized by multilocus sequence typing. The 11 STs were grouped into 6 different clonal complexes: ST103 complex (n = 7), ST11 complex (n = 5), ST213 complex (n = 1), ST32 complex (n = 3), ST41/44/Lineage 3 (n = 1), ST461 (n = 1). The most prevalent clonal complex, ST103 complex, was represented by ST3780 (n = 6) (Table 2).

We did not find an increased risk of meningococci carriage associated with any of the potential risk factors studied, except low level of education. A low education level (i.e., not completing secondary education) was significantly associated with a higher risk for carriage of meningococci, regardless of serogroup identification (Table 3).

Conclusions

Most published studies report a consistently low rate (usually <1%) of MenC carriage during outbreaks of MenC disease (*14–16*). However, after outbreaks at 2 oil refineries in São Paulo State, Brazil, we found high rates (6.3% and 4.9%, respectively) of MenC carriage among refinery workers.

Mass vaccination with a meningococcal A/C polysaccharide vaccine was conducted at Refinery A, and high coverage (91%) was achieved among workers. This intervention controlled the MenC outbreak in the refinery; only 1 new case occurred after the vaccination campaign, but that case cannot be considered the result of a vaccine failure because it occurred <14 days after the refinery worker was vaccinated. These findings likely indicate that the workers received direct protection against MenC from vaccine. However, after the vaccination campaign, 9 new cases of MenC infection occurred in children who were household contacts of vaccinated workers, without any known contact among them.

The prevalence of MenC carriage was high among workers at both refineries, even though 91% of Refinery A workers had received meningococcal A/C polysaccharide vaccine 6 months before our study began. More striking, carriage rates among vaccinated and nonvaccinated workers were similar. These findings suggest that meningococcal A/C polysaccharide vaccine had no effect on MenC carriage. Most of the studies conducted among nonmilitary populations demonstrated that these vaccines cannot significantly reduce meningococcal carriage (*17–20*). The short-term persistence of circulating antibodies and the quality of the immune response induced after vaccination with a polysaccharide vaccine may partly explain why these vaccines have no effect on carriage (*20–24*).

In contrast to polysaccharide vaccines, conjugate vaccines lead to the production of very high antibody concentrations, even in infants, and induce immunologic memory with higher antibody avidity and increased serum bactericidal activity, thus providing more robust long-term protection. In addition, conjugate vaccines also prevent the acquisition of carriage among vaccinees and, by interrupting transmission, provide indirect protection to unvaccinated, susceptible persons; this herd immunity proved key to the success of MCC vaccination programs in various countries (*25–27*).

Table 1. Pharyngeal carriage of *Neisseria meningitidis* among vaccinated and nonvaccinated workers at 2 oil refineries, São Paulo, Brazil, 2010*

N. meningitidis serogroup	No. (%) workers			p value‡
	Refinery A*	Refinery B†	Total	
All	51 (21.4)	53 (21.6)	104 (21.5)	1.00
C	15 (6.3)	12 (4.9)	27 (5.6)	0.64
B	4 (1.6)	5 (2.0)	9 (1.9)	1.00
W	4 (1.6)	1 (0.4)	5 (1.0)	0.35
Y	5 (2.1)	2 (0.8)	7 (1.4)	0.43
E	3 (1.2)	5 (2.0)	8 (1.7)	0.76
Nongroupable	20 (8.4)	28 (11.4)	48 (9.9)	0.34
Negative	187 (78.6)	192 (78.4)	379 (78.5)	
Total	238 (100.0)	245 (100.0)	483 (100.0)	

*Vaccinated workers.
†Unvaccinated workers.
‡By Fisher exact test.

Table 2. Phenotypic and genotypic characteristics of *Neisseria meningitidis* strains isolated from nasopharyngeal samples of workers at 2 oil refineries, São Paulo, Brazil, 2010*

Refinery, *N. meningitidis* serogroup, worker's age, y	Serotype:serosubtype	ST	Clonal complex
A			
B			
20	4,7:NST	9858	
29	19,1:P1.14	6481	ST213 complex
19	17:P1.5	8035	ST41/44 complex/Lineage 3
C			
26	23:P1.14–6	3780	ST103 complex
28	23:P1.14–6	8730	NA
21	23:P1.14–6	8730	NA
22	23:P1.14–6	8730	NA
20	23:P1.14–6	8730	NA
W			
21	2b:P1.2	11	ST11 complex/ET-37 complex
27	2b:P1.5,2	11	ST11 complex/ET-37 complex
24	2b:P1.5,2	11	ST11 complex/ET-37 complex
Y			
23	2a:P1.5,2	11	ST11 complex/ET-37 complex
26	17,7:P1.5	6525	NA
22	17,7:P1.5	6525	NA
B			
B			
26	19,1:NST	1869	ST461 complex
19	4,7:P1.19,15	7594	ST32 complex/ET-5 complex
28	4,7:P1.19,15	7594	ST32 complex/ET-5 complex
C			
25	23:P1.14–6	3780	ST103 complex
21	23:P1.14–6	3780	ST103 complex
25	23:NST	3780	ST103 complex
28	23:P1.5	3779	ST103 complex
23	4,7:P1.19,15	3773	ST32 complex/ET-5 complex
28	23:P1.14–6	3780	ST103 complex
25	23:P1.14–6	8730	NA
23	23:P1.14–6	3780	ST103 complex
W			
26	2b:P1.2	11	ST11 complex/ET-37 complex
Y			
19	19,7:P1.5	6525	NA

*NST, not serosubtypeable; ST, sequence type; NA, assigned without clonal complex.

The characterization of the *N. meningitidis* strains isolated from the patients (workers and family contacts) during the outbreak in Refinery A has been described (*28*). The characterization showed that all MenC isolates were genetically related and displayed the same phenotype, C:23:P1.14–6, associated with ST3780 of the ST103 complex. The characterization of the 13 MenC carriage strains recovered from workers at both refineries in our study showed that most (10/13) displayed the C:23:P1.14–6 phenotype. These strains displayed 2 STs: ST3780, which belongs to ST103 complex, and ST8730, assigned without clonal complex. In Brazil, the increase in MenC disease during the last decade has been associated with the emergence of this virulent clone belonging to the ST103 complex (*2*,*29*). The ability of MCC vaccines to effect carriage of strains from the ST103 complex has yet to be shown. The recent introduction of MCC vaccine in the routine immunization program in Brazil will provide this opportunity, highlighting the importance of

Table 3. Risk factors for pharyngeal carriage of *Neisseria meningitidis* among workers at 2 oil refineries, São Paulo, Brazil, 2010

	All *N. meningitidis* strains			Serogrouped *N. meningitidis* strains		
Variable	% Workers exposed	% Workers not exposed	p value*	% Workers exposed	% Workers not exposed	p value*
Antimicrobial drug use†	12.9	22.1	0.16	3.2	12.0	0.11
Crowded living conditions	17.4	22.9	0.14	9.9	12.3	0.35
Active smoking	23.2	21.2	0.41	11.6	11.6	0.58
Respiratory symptoms†	24.2	20.9	0.26	10.1	11.9	0.44
Low level of education‡	32.9	19.2	0.01	17.0	10.6	0.07

*By Fisher exact test.
†In the 15 d before the collection of the nasopharyngeal sample.
‡Defined as not completing secondary education.

carefully designed studies to measure the effect of the vaccine on carriage and transmission.

Meningococcal carriage was not associated with any of the risk factors evaluated in our study, except the level of education, which was inversely related to the prevalence of carriage. The higher percentage of MenC carriers among study participants with a lower level of education presumably reflects associated socioeconomic conditions and social behaviors. Less-educated workers in oil refinery settings are also more likely to perform activities that require the use of ear devices as protection from the loud environment. The wearing of such devices forces workers to stay very close to each other to facilitate conversation among them, and such close working situations also facilitate transmission of meningococci.

Although the relationship between meningococci carriage prevalence and disease incidence is not fully understood, the evidence gathered during this study showed a dominance of the C:23:P1.14–6 phenotype strain among workers from both refineries, reinforcing the concept that the dominance of a particular strain is an important marker of epidemic conditions (*30*,*31*). Also, in accordance with previous findings from other studies, we observed that polysaccharide vaccination had no effect on carriage and did not interrupt transmission to susceptible contacts (*4*,*24*). These results represent a challenge to the current policy of using the meningococcal polysaccharide A/C vaccine to control outbreaks of MenC disease, and they have key implications for future vaccination strategies. Our findings emphasize the need to review such policies and to consider using MCC vaccines rather than meningococcal polysaccharide A/C vaccines to control MenC disease outbreaks.

Acknowledgments

We thank Marcelle Viçoso dos Santos, Conceição Zanelato, Maria Vaneide de Paiva, and Martha Galhardo for assistance in conducting microbiological tests.

An unrestricted education grant from Sanofi Pasteur vaccines was provided for this study.

M.A.P.S. has been a scientific consultant and a speaker for GlaxoSmithKline, Novartis, Wyeth/Pfizer, Sanofi Pasteur, and MSD (MerckSharpDohme); J.C.M. has been a scientific consultant for Novartis, Wyeth/Pfizer, and Sanofi Pasteur. M.C.C.B. has been a speaker for Sanofi-Pasteur, Pfizer, and Glaxo Smith Kline. A.P.L. has been a speaker for Novartis and Sanofi Pasteur. M.C.O.G. has been a speaker for Novartis.

Dr Sáfadi is a pediatric infectious disease specialist and an assistant professor in the Department of Pediatrics at Faculdade de Ciências Médicas da Santa Casa, São Paulo. His primary research interests include epidemiology and prevention of childhood infectious diseases.

References

1. Brazilian Ministry of Health. Health surveillance [cited 2012 Aug 20]. http://portal.saude.gov.br/portal/saude/area.cfm?id_area=962.
2. Sáfadi MA, González-Ayala S, Jäkel A, Wieffer H, Moreno C, Vyse A. The epidemiology of meningococcal disease in Latin America 1945–2010: an unpredictable and changing landscape. Epidemiol Infect. 2013;141:447–58 http://dx.doi.org/10.1017/S0950268812001689
3. Centro de Vigilância Epidemiológica. "Prof. Alexandre Vranjac" (Epidemiologic Surveillance Center), Brazil. Respiratory transmitted diseases [in Portuguese] [cited 2012 Aug 30]. http://www.cve.saude.sp.gov.br/htm/resp/dm_soro.htm
4. Iser BPM, Lima CAV, de Moraes C, de Almeida RP, Watanabe LT, Alves SL, et al. Outbreak of *Neisseria meningitidis* C in workers at a large food-processing plant in Brazil: challenges of controlling disease spread to the larger community. Epidemiol Infect. 2012;140:906–15. http://dx.doi.org/10.1017/S0950268811001610
5. Gorla MC, de Lemos AP, Quaresma M, Vilasboas R, Marques O, de Sá MU, et al. Phenotypic and molecular characterization of serogroup C *Neisseria meningitidis* associated with an outbreak in Bahia, Brazil. Enferm Infecc Microbiol Clin. 2012;30:56–9. http://dx.doi.org/10.1016/j.eimc.2011.07.022
6. Puricelli RCB, Kupek E, Bertoncini RCC. Control of a community outbreak of group C meningococcal meningitis in Corupá, Santa Catarina State, Brazil, based on a rapid and effective epidemiological surveillance and immunization [in Portuguese]. Cad Saude Publica. 2004;20:959–67 http://dx.doi.org/10.1590/S0102-311X2004000400010
7. O'Brien KL, Brondson MA, Dagan R, Yagupsky P, Janco J, Elliott J, et al. Evaluation of a medium (STGG) for transport and optimal recovery of *Streptococcus pneumoniae* from nasopharyngeal secretions collected during field studies. J Clin Microbiol. 2001;39:1021–4. http://dx.doi.org/10.1128/JCM.39.3.1021-1024.2001
8. World Health Organization Communicable Disease Surveillance and Response. Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis, Streptococcus pneumoniae*, and *Haemophilus influenzae*. WHO/CDS/CSR/EDC/99.7; 1998 [cited 2012 Nov 7]. http://www.who.int/csr/resources/publications/meningitis/whocdscsredc997.pdf.
9. Alkmin MG, Shimizu SH, Landgraf IM, Gaspari EN, Melles CE. Production and immunochemical characterization of *Neisseria meningitidis* group B antiserum for the diagnosis of purulent meningitis. Braz J Med Biol Res. 1994;27:1627–34.
10. Mothershed EA, Sacchi CT, Whitney AM, Barnett GA, Ajello GW, Schmink S. Use of real-time PCR to resolve slide agglutination discrepancies in serogroup identification of *Neisseria meningitidis*. J Clin Microbiol. 2004;42:320–8. http://dx.doi.org/10.1128/JCM.42.1.320-328.2004
11. Dolan Thomas J, Hatcher CP, Satterfield DA, Theodore MJ, Bach MC, Linscott KB, et al. *sodC*-based real-time PCR for detection of *Neisseria meningitidis*. PLoS ONE. 2011;6:e19361. http://dx.doi.org/10.1371/journal.pone.0019361
12. Wedege E, Hoiby EA, Rosenqvist E, Froholm LO. Serotyping and subtyping of *Neisseria meningitidis* isolates by co-agglutination, dot-blotting and ELISA. J Med Microbiol. 1990;31:195–201. http://dx.doi.org/10.1099/00222615-31-3-195
13. Maiden MC, Bygraves JA, Feil E, Morelli G, Russel JE, Zhang Q, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A. 1998;95:3140–5. http://dx.doi.org/10.1073/pnas.95.6.3140
14. Jones GR, Williams JN, Christodoulides M, Jolley K, Heckels JE. Lack of immunity in university students before an outbreak of serogroup C meningococcal infection. J Infect Dis. 2000;181:1172–5. http://dx.doi.org/10.1086/315352

15. Conyn-van Spaendonck MA, Reintjes R, Spanjaard L, van Kregten E, Kraaijeveld AG, Jacobs PH. Meningococcal carriage in relation to an outbreak of invasive disease due to *Neisseria meningitidis* serogroup C in the Netherlands. J Infect. 1999;39:42–8. http://dx.doi.org/10.1016/S0163-4453(99)90101-9
16. Patrick DM, Champagne S, Goh SH, Arsenault G, Thomas E, Shaw C, et al. *Neisseria meningitidis* carriage during an outbreak of serogroup C disease. Clin Infect Dis. 2003;37:1183–8. http://dx.doi.org/10.1086/378743
17. Ramos Aceitero JM. Surveys on the rates of healthy carriers of *Neisseria meningitidis* and characterization of circulating strains. Rev Esp Salud Publica. 2000;74:413–7.
18. Cardeñosa N, Domínguez A, Orcau A, Pañella H, Godoy P, Minguell S, et al. Carriers of *Neisseria meningitidis* in household contacts of meningococcal disease cases in Catalonia (Spain). Eur J Epidemiol. 2001;17:877–84. http://dx.doi.org/10.1023/A:1015696513062
19. Domínguez A, Cardeñosa N, Izquierdo C, Sánchez F, Margall N, Vazquez JA, et al. Prevalence of *Neisseria meningitidis* carriers in the school population of Catalonia, Spain. Epidemiol Infect. 2001;127:425–33. http://dx.doi.org/10.1017/S0950268801006173
20. Poland GA. Prevention of meningococcal disease: current use of polysaccharide and conjugate vaccines. Clin Infect Dis. 2010;50(Suppl 2):S45–53. http://dx.doi.org/10.1086/648964
21. Granoff DM, Harrison LH, Borrow R. Meningococcal vaccines. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines. 5th ed. London: Saunders-Elsevier; 2008. p. 399–434.
22. Stephens DS. Conquering the meningococcus. FEMS Microbiol Rev. 2007;31:3–14 http://dx.doi.org/10.1111/j.1574-6976.2006.00051.x
23. Danzig L. Meningococcal vaccines. Pediatr Infect Dis J. 2004;23 (Suppl):S285–92.
24. Granoff DM, Pollard AJ. Reconsideration of the use of meningococcal polysaccharide vaccine. Pediatr Infect Dis J. 2007;26:716–22. http://dx.doi.org/10.1097/INF.0b013e3180cc2c25
25. Maiden MC, Ibarz-Pavón AB, Urwin R, Gray SJ, Andrews NJ, Clarke SC, et al. Impact of meningococcal serogroup C conjugate vaccines on carriage and herd immunity. J Infect Dis. 2008;197:737–43. http://dx.doi.org/10.1086/527401
26. Sáfadi MA, McIntosh ED. Epidemiology and prevention of meningococcal disease: a critical appraisal of vaccine policies. Expert Rev Vaccines. 2011;10:1717–30. http://dx.doi.org/10.1586/erv.11.159
27. Trotter CL, Maiden MC. Meningococcal vaccines and herd immunity: lessons learned from serogroup C conjugate vaccination programs. Expert Rev Vaccines. 2009;8:851–61. http://dx.doi.org/10.1586/erv.09.48
28. Liphaus BL, Cappeletti-Gonçalves-Okai MI, Silva-Delemos AP, Gorla MC, Rodriguez-Fernandes M, Pacola MR, et al. Outbreak of *Neisseria meningitidis* C in a Brazilian oil refinery involving an adjacent community. Enferm Infecc Microbiol Clin. 2013;31:88–92. http://dx.doi.org/10.1016/j.eimc.2012.05.009.
29. de Lemos AP, Yara TY, Gorla MC, de Paiva MV, de Souza AL, Gonçalves MIC, et al. Clonal distribution of invasive *Neisseria meningitidis* serogroup C strains circulating from 1976 to 2005 in greater São Paulo, Brazil. J Clin Microbiol. 2007;45:1266–73. http://dx.doi.org/10.1128/JCM.02510-06
30. Trotter CL, Greenwood BM. Meningococcal carriage in the African meningitis belt. Lancet Infect Dis. 2007;7:797–803. http://dx.doi.org/10.1016/S1473-3099(07)70288-8
31. Raghunathan PL, Jones JD, Tiendrebéogo SR, Sanou I, Sangaré L, Kouanda S, et al. Predictors of immunity after a major serogroup W-135 meningococcal disease epidemic, Burkina Faso, 2002. J Infect Dis. 2006;193:607–16. http://dx.doi.org/10.1086/499822

Address for correspondence: Marco A. P. Sáfadi, Faculdade de Ciências Médicas da Santa Casa, Alameda dos Indigenas, 228 ZIP 04059-060, São Paulo, Brazil; email: masafadi@uol.com.br

Treatment Practices, Outcomes, and Costs of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis, United States, 2005–2007

Suzanne M. Marks, Jennifer Flood, Barbara Seaworth, Yael Hirsch-Moverman, Lori Armstrong, Sundari Mase, Katya Salcedo, Peter Oh, Edward A. Graviss, Paul W. Colson, Lisa Armitige, Manuel Revuelta, Kathryn Sheeran, and the TB Epidemiologic Studies Consortium

To describe factors associated with multidrug-resistant (MDR), including extensively-drug-resistant (XDR), tuberculosis (TB) in the United States, we abstracted inpatient, laboratory, and public health clinic records of a sample of MDR TB patients reported to the Centers for Disease Control and Prevention from California, New York City, and Texas during 2005–2007. At initial diagnosis, MDR TB was detected in 94% of 130 MDR TB patients and XDR TB in 80% of 5 XDR TB patients. Mutually exclusive resistance was 4% XDR, 17% pre-XDR, 24% total first-line resistance, 43% isoniazid/rifampin/rifabutin-plus-other resistance, and 13% isoniazid/rifampin/rifabutin-only resistance. Nearly three-quarters of patients were hospitalized, 78% completed treatment, and 9% died during treatment. Direct costs, mostly covered by the public sector, averaged $134,000 per MDR TB and $430,000 per XDR TB patient; in comparison, estimated cost per non-MDR TB patient is $17,000. Drug resistance was extensive, care was complex, treatment completion rates were high, and treatment was expensive.

Author Affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (S.M. Marks, L. Armstrong, S. Mase, TB Epidemiologic Studies Consortium); California Department of Public Health, Richmond, California, USA (J. Flood, K. Salcedo, P. Oh); Texas Department of State Health Services, Tyler, Texas, USA (B. Seaworth, L. Armitige, K. Sheeran); University of Texas Health Science Center, Tyler (B. Seaworth, L. Armitige, K. Sheeran); ICAP/Columbia University, New York, New York, USA (Y. Hirsch-Moverman, P.W. Colson, M. Revuelta); and Methodist Hospital Research Institute, Houston, Texas, USA (E.A. Graviss)

DOI: http://dx.doi.org/10.3201/eid2005.131037

Drug-resistant *Mycobacterium tuberculosis* poses substantial obstacles to tuberculosis (TB) control. In the United States, multidrug-resistant (MDR) TB (resistant to at least isoniazid and rifampin) comprises only 1.0%–1.5% of TB cases but requires lengthy regimens of toxic drugs, imposes high costs on the health care system and society, and causes high mortality rates.

Studies of MDR TB in the United States have been limited by small sample sizes, limited study periods, minimal information on outcomes and costs, or reliance solely on surveillance data (*1*–*6*), which omit some cases of acquired drug resistance and changes in regimens. Costs of treating MDR TB are not routinely collected or reported.

Our study describes and analyzes characteristics associated with drug resistance, timely diagnosis, treatment practices, outcomes, and costs associated with MDR TB for cases reported to the Centers for Disease Control and Prevention (CDC) by California, New York, and Texas during 2005–2007. These 3 areas contribute about half of US MDR TB cases annually.

Methods

CDC and local institutional review boards approved the study and granted a waiver of patient informed consent and patient authorization. We defined a 5-drug regimen (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1037-Techapp1.pdf) to be consistent with US and World Health Organization recommendations (*7*,*8*). All study definitions are in the online Technical Appendix.

Each site identified cases of MDR TB and extensively drug-resistant (XDR) TB reported to CDC during

2005–2007. The study included all XDR TB cases and a 75% simple random sample of MDR TB cases from California and New York (New York City), and a 50% sample from Texas. Using standardized forms, we abstracted hospital, laboratory, and public health clinic records retrospectively for patient demographic, socioeconomic, and clinical characteristics and for treatment, case management, outcomes, and costs. Total charges for each TB-associated hospitalization were abstracted from hospital UB-04 forms. To ascertain sputum-culture conversion and drug resistance, we examined all available culture and drug-susceptibility testing (DST) results from diagnosis through treatment.

To assess representativeness, we compared our sample with National TB Surveillance System data from all US sites. We identified characteristics among MDR TB patients associated with the following 3 dichotomous outcomes: drug resistance acquisition, expert consultation use, and death during TB treatment. Multivariable logistic regression was used with backward selection at $p<0.05$ to identify variables remaining in final models (SAS version 9.2/9.3; SAS Institute, Cary, NC, USA). Adjusted odds ratios (AORs) significant at 95% CIs and Schwarz Criterion statistics are reported for goodness-of-fit. Variables included in initial models were patient demographics (gender, age group, race/ethnicity, foreign birth), socioeconomic factors (homelessness, unemployment, illicit substance use, excess alcohol use, smoking), medical risks (HIV infection, diabetes), TB history, disease severity (acid-fast bacilli [AFB]–smear positivity, cavitation, dissemination), drug-resistance pattern, receipt of TB clinic outpatient care, and additional relevant characteristics (for acquired resistance: receipt of ≥4 effective medications; for expert consultation: incarceration in a correctional institution, long-term-care facility residence, pregnancy, death during treatment, number of adverse events, private outpatient insurance; for death during treatment: pregnancy, incarceration in a correctional institution).

Inpatient costs were measured; outpatient costs and productivity losses were estimated (online Technical Appendix). Hospital charges were converted to costs by using hospital-specific operating cost-to-charge ratios (*9*). All costs were converted to 2010 US dollars (*10*) and were adjusted for cost of living (1.13 for California, 1.08 for New York City, and 0.94 for Texas) (*11*) to facilitate aggregation. For 17 patients for whom hospital charges data were missing, we multiplied hospitalization duration by average cost per day for patients for whom data were available ($1,419).

Study total direct costs were compared with estimated direct costs for cases of non-MDR TB (online Technical Appendix). We report SEMs to display cost variability.

For productivity losses from hospitalization, we applied an updated 2010 dollar value of work-plus-home production of $224/day for employed patients and $40/day for home-only production of unemployed patients (*12*). For TB-related deaths, we estimated the value of remaining lifetime productivity, updated to 2010 dollars, based on the age at death (*12*). For patients experiencing adverse events during treatment, we calculated a disability adjustment per patient (100%, 83%, 67%, 50%, 33%, 17%, 0%). We estimated direct and productivity-loss costs and examined associated characteristics by using multivariable linear regression with backward selection. Adjusted R^2 statistics are reported to show goodness-of-model-fit.

Results

The sample consisted of 135 patients (130 with MDR TB and 5 with XDRTB), representing 36% (130/364) of MDR TB and 56% (5/9) of XDR TB cases reported in the United States during 2005–2007. Among these patients, 87% were foreign born and 36% had prior TB disease (Tables 1–3). Among patients for whom information about concurrent medical conditions was available, 24 (20%) of 121 had diabetes and 14 (12%) of 116 had HIV infection. The study population resembled all US MDR TB patients; however, fewer study participants were White or unemployed, and more used noninjection drugs, had prior TB, or had AFB-positive smear specimens. Similar to all foreign-born TB patients in the United States, most foreign-born MDR TB study participants arrived from Mexico, the Philippines, India, and Vietnam. Of the 135 patients, 7% were homeless before diagnosis (6 patients) or during treatment (3 patients). Case management to obtain housing during treatment was needed by 23 (17%) patients; 38 (28%) patients had been unemployed before diagnosis, and of the 97 remaining patients, 27% stopped work because of MDR TB.

Of the 135 patients, disease was pulmonary for 85%, extrapulmonary only for 6%, and disseminated for 9%. Of 127 patients with pulmonary or disseminated disease, 69 (54%) had extensive disease. Of 8 patients with extrapulmonary-only disease, 2 (25%) had extensive disease. Of 134 patients alive at diagnosis, 77% (103) had at least 1 AFB-smear–positive specimen, 72% from sputum.

Drug Resistance

At various times during treatment, *M. tuberculosis* isolates were tested for susceptibility (median 14 medications, range 4–19) and were resistant to several (median 5 medications, range 2–16) at any time during treatment. The following mutually exclusive resistance patterns were identified: 4% XDR, 17% pre-XDR, 24% total first-line resistance, 43% isoniazid/rifampin/rifabutin-plus-other resistance, and 13% isoniazid/rifampin/rifabutin-only resistance (Figure 1). Initial *M. tuberculosis* isolates obtained within 30 days of treatment initiation revealed isoniazid/rifampin resistance among 122 (94%) of the 130 MDR TB patients and XDR among 4 (80%) of the 5 XDR TB patients. DST

was conducted for first-line drugs during the first month of treatment, for second-line drugs during the second month of treatment, for linezolid during the fourth month, and for clofazimine during the seventh month.

Among 128 patients for whom DST was conducted multiple times on separate dates, acquired resistance to an anti-TB medication during treatment was detected for 27 (21%). According to multivariable analysis, acquisition of drug resistance during treatment was more likely for patients who were recently homeless (AOR 5.8, 95% CI 1.2–28.9), who had pre-XDR or XDR TB (AOR 5.1, 95% CI 1.9–14.2), or who were Black (AOR 4.1, 95% CI 1.1–15.4) (Table 4). Acquisition of resistance to isoniazid or rifampin, resulting in MDR TB, occurred for 6% of patients, to fluoroquinolones for 3%, and to injectable drugs for 4%. Other medications to which resistance was acquired were ethambutol (10% of patients), pyrazinamide (6%), streptomycin (5%), ethionamide (5%), rifabutin (2%), cycloserine (1%), and clofazimine (1%). For 1 patient, fluoroquinolone resistance was acquired, resulting in XDR TB.

Time to Diagnosis

For 74 patients who had TB symptoms before diagnosis, a median of 1.6 months elapsed from symptom onset to initial TB diagnosis. Of 134 patients alive at diagnosis, 123 (92%) started receiving ≥2 second- or third-line medications a median of 2.4 months after initial TB diagnosis. The median duration of infectiousness was 10 months.

Treatment Practices

Among 105 patients who completed treatment, treatment duration varied. Median durations were 32.3 months (interquartile range [IQR] 30.6–37.8) for those with XDR, 25.1 months (IQR 23.6–29.2) for those with pre-XDR, 25.7 months (IQR 22.4–26.9) for those with total-first-line–resistant, 24.1 months (IQR 20.1–27.0) for those with isoniazid/rifampin/rifabutin-plus–resistant, and 20.0 months (IQR 19.4–24.5) for those with isoniazid/rifampin/rifabutin-only–resistant TB.

Providers changed medications for 134 patients alive at diagnosis a median of 7 times during treatment; 33% of 988 medication changes were because of adverse events and 10% because of DST results. Of 134 patients, 34% received a 5-drug regimen before sputum-culture conversion, and 61% ultimately received a 5-drug regimen. Of the 134 patients, 81% received an effective (i.e., medication to which their isolate was never resistant) injectable medication and 86% received an effective fluoroquinolone medication during treatment. Of 123 patients who received outpatient care, 90% received >80% of outpatient medication doses by directly observed therapy (DOT).

Most study patients were hospitalized for TB, often several times; 73% (98) were hospitalized at least 1 time and 29% were hospitalized ≥2 times (range 2–6). Detailed data were available for 83% of inpatients. Among multiple possible reasons, severe worsening of TB disease was the reason for 50% of hospitalizations, followed by the need to initiate or change treatment (40%), implement respiratory isolation (21%), manage adverse events (7%), manage concurrent conditions (3%), and perform surgery (1%). One patient had undergone TB-related lung lobectomy. Four XDR TB patients were hospitalized for a median of 282 days (range 14–850) and non-XDR patients for a median of 27 days (range 1–759). Home isolation was prescribed for 37% of patients; a median of 102 days (range 4–337) for non-XDR TB patients and 257 days for the 1 XDR TB patient.

Table 1. Demographic characteristics of study participants and all patients with MDR and/or XDR TB, United States, 2005–2007*

Characteristic	Study participants, no. (%), n = 135	All US MDR TB patients, no. (%), n = 370
Sex		
M	68 (50)	205 (55)
F	67 (50)	164 (44)
Age, y		
Median	38.2	
0–14	1 (1)	13 (4)
15–24	24 (18)	64 (17)
25–44	62 (46)	171 (46)
45–64	40 (30)	91 (25)
≥65	8 (6)	31 (8)
Race/ethnicity		
Hispanic	42 (31)	100 (27)
White†	4 (3)	31 (8)
Black	14 (10)	63 (17)
Asian	72 (53)	173 (47)
Other/unknown	3 (2)	3 (1)
Geographic origin		
Foreign born‡	118 (87)	305 (82)
US born	17 (13)	64 (17)

*Study patients were from California, New York City, and Texas. MDR, multiple-drug resistant; XDR, extensively drug resistant; TB, tuberculosis.
†Statistically significant differences between percentages of study patients and all US MDR TB patients at p<0.05.
‡For 110 participants, median no. years after first entry into United States = 3.5.

Table 2. Socioeconomic characteristics of study participants and all patients with MDR and/or XDR TB, United States, 2005–2007*

Characteristic	Study participants, no. (%), n = 135	All US MDR TB patients, no. (%), n = 370
Unemployed†	38 (28)	187 (51)
Homeless	9 (7)	20 (5)
Correctional institution resident	6 (4)	7 (2)
Long-term care facility resident	4 (3)	6 (2)
Injection drug use	5 (4)	8 (2)
Noninjection drug use†	12 (9)	15 (4)
Excess alcohol use	15 (11)	31 (8)
Smoker	31 (23)	
Pregnant at treatment initiation	6 (4)	
Private health insurance, % of 112 known	24 (21)	
Public health insurance, % of 112 known	49 (44)	
Jail/prison health coverage, % of 112 known	2 (2)	
Other health insurance, % of 112 known	5 (4)	
No health insurance, % of 112 known	32 (29)	
HIV+	14 (10)	29 (8)
Receiving HAART	9 (64)	
Receiving ART	1 (7)	
Receiving neither HAART nor ART	3 (21)	
Receipt of ART not documented	1 (7)	
Not HIV infected	102 (76)	205 (55)
HIV status unknown	19 (14)	136 (37)
Diabetes, % of 121 known	24 (20)	
ESRD, % of 121 known	3 (2)	
Prolonged corticosteroid therapy, % of 121 known	2 (2)	
Other immunosuppressive therapy, % of 121 known	2 (2)	
Cancer, % of 121 known	3 (2)	
Hematologic diseases, % of 121 known	2 (2)	

*Study patients were from California, New York City, and Texas. MDR, multidrug resistant; XDR, extensively drug resistant; TB, tuberculosis; HIV+, HIV infected; HAART, highly active antiretroviral therapy; ART, antiretroviral therapy; ESRD, end-stage renal disease. Blank cells indicate data not available.
†Statistically significant differences between percentages of study patients and all US MDR TB patients at p<0.05.

Of the 134 patients alive at diagnosis, 81% had documentation of physician consultation with an MDR TB expert during inpatient or outpatient care. Expert consultation was more likely for patients managed primarily by a TB clinic (AOR 5.7, 95% CI 1.9–16.8) and less likely for those with private insurance (AOR 0.2, 95% CI = 0.1–0.7) (Table 5). Overall, each patient received a median of 3 expert consultations.

Of the 134 patients alive at diagnosis, ≈90% were assigned a case manager. Case management activities included home visits (68%), social worker assistance (37%), transportation assistance (32%), incentives (25%), housing assistance (17%), and other activities including legal orders for DOT or isolation (9%–15%). Only 4% of patients received none of these case management services. Interpreter use was documented for 60% of 107 patients who understood some or no English.

Outcomes

Of 112 eligible patients, including all XDR TB patients, sputum culture converted to negative for 109 (97%). Patients considered ineligible for culture conversion included 1 patient whose TB was diagnosed after death, 6 who died during treatment, 3 who were transferred to another US jurisdiction or out of the United States, 7 who had extrapulmonary-only disease, and 6 without a positive sputum culture result. Of the 3 for whom no culture conversion was documented, 1 was lost to follow-up after 166 days and 2 completed treatment. Culture conversion occurred within a median of 2 months from starting a 5-drug-regimen but varied by resistance pattern (online Technical Appendix).

Of the 134 patients alive at diagnosis, 78% completed treatment, 11% transferred within or outside the United States or were lost to follow-up, and 1% stopped treatment because of adverse events (Table 6). For no patients did treatment fail or TB recur within the year after treatment completion. Of the 134 patients, 12 (9%) died during treatment; 75% of these deaths were considered TB related. No XDR TB patient died. Death during treatment was significantly associated with age ≥65 years (AOR 20.2, 95% CI 2.3–181.0), smoking (AOR 6.4, 95% CI 1.0–39.4), or HIV infection (AOR 6.3, 95% CI 1.1–37.7) (online Technical Appendix). When TB medications and interaction terms were initially included in the model, HIV infection was no longer associated with death and receipt of an effective injectable medication was associated with lower odds of dying (AOR 0.02, 95% CI 0.002–0.2) (online Technical Appendix). No HIV-infected patient who received an effective injectable medication died. Of 9 patients who died of TB-related causes, only 2 who had received a 5-drug regimen died, both after 8 months; 1 had received 3 non–first-line medications and died after 5 months, and the remaining 6 never received ≥2 MDR TB second- or third-line medications and died within 49 days of treatment initiation.

Table 3. Clinical characteristics of study participants and other patients with MDR and/or XDR TB, United States, 2005–2007*

Characteristic	Study participants, no. (%), n = 135	All US MDR TB patients, no. (%), n = 370
History of LTBI, % of 130 known	21 (16)	
History of completing LTBI Rx, % of 21 with history of LTBI	14 (67)	
History of TB disease†	48 (36)	60 (16)
Contact with infectious TB patient, % of 87 known	10 (11)	
Contact with infectious MDR TB patient, % of 10 contacts	6 (60)	
Dead at TB diagnosis	1 (1)	5 (1)
Smear positive at any time, % of 134 alive at diagnosis†	103 (77)	223 (60)
Sites of TB disease		
Pulmonary	115 (85)	332 (90)
Extrapulmonary only	8 (6)	37 (10)
Disseminated at any time	12 (9)	
Extent of pulmonary disease at diagnosis		
Extensive	69	
Moderate	31	
Minimal	21	
Undocumented	6	
Description of extensive pulmonary disease, at any time		
Miliary	4	
Cavitary	58	127
Multiple lobes	70	
Collapsed lobes	6	
Extent of extrapulmonary disease at diagnosis		
Extensive	2	
Moderate	3	
Minimal	3	

*MDR, multidrug resistant; XDR, extensively drug resistant; TB, tuberculosis; LTBI, latent TB infection; Rx, treatment. Blank cells indicate data not available.
†Statistically significant differences between percentages of study patients and all US MDR TB patients at p<0.05.

Of the 4 HIV-infected patients who died of TB-related causes, 3 had a CD4 count of ≤50 cells/mm^3 at initiation of TB treatment and the other had a CD4 count of ≤100 cells/mm^3. Of these 4 patients, 2 were taking ≥3 anti-HIV medications.

Among patients alive at diagnosis, a median of 1 adverse event (average 2.9) resulted in medication change or adjustment, but only 2 patients completely stopped treatment. According to multivariate linear regression, use of clofazimine was significantly associated (p<0.05) with more adverse events but was not associated with death during treatment. Of 9 patients receiving clofazimine, 6 experienced postinitiation gastrointestinal effects that resulted in their discontinuing clofazimine. Because of MDR TB or its treatment, of the 134 patients, 13% experienced hearing impairment, 13% hepatitis, 11% renal impairment, 8% difficulty ambulating, 7% visual impairment, and 1% seizures. Depression or psychosis was documented for 19% (80% of whom were taking cycloserine), and pulmonary impairment was documented for 4%. Of 103 impairments, 66% were mild, but another 7% were graded most severe and occurred for 6 patients, 3 of whom completed treatment; 2 of those patients died (lung and mobility impairments), and 1 transferred outside the country.

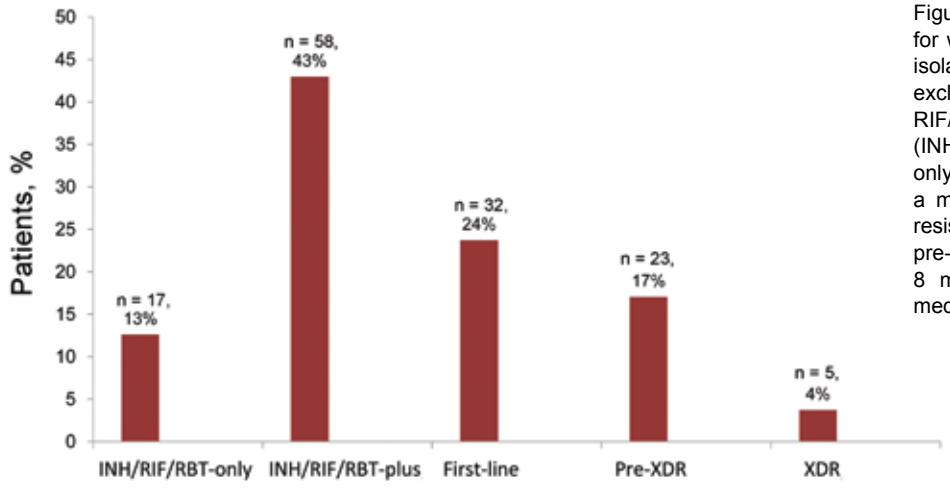


Figure 1. Percentage of 135 patients for whom *Mycobacterium tuberculosis* isolates had the following mutually exclusive resistance patterns. INH/RIF/RBT-only, resistant to isoniazid (INH)/rifampin (RIF)/rifabutin (RBT) only; INH/RIF/RBT-plus, resistant to a median of 4 medications; first-line, resistant to a median of 6 medications; pre-XDR, resistant to a median of 8 medications; XDR, resistant to a median of 11 medications.

Table 4. Characteristics associated with any acquired antimicrobial drug resistance during MDR TB treatment, 128 patients, California, Texas, and New York, NY, USA, 2005–2007*†

Variable	Initial OR estimate	Initial 95% CI	Initial Pr>χ^2	Final OR estimate	Final 95% CI	Final Pr>χ^2
Age ≥65 y	<0.001	<0.001–>999.999	0.973			
Black race	33.19	0.80–>999.999	0.065	**4.07**	**1.08–15.37**	**0.039**
Recent homelessness	18.76	0.93–377.71	0.056	**5.81**	**1.17–28.86**	**0.031**
Pre-XDR or XDR TB	8.78	2.31–33.42	0.001	**5.15**	**1.86–14.21**	**0.002**
AFB-smear positive	5.34	0.86–33.22	0.072			
Age 25-44 y	4.83	0.65–36.03	0.124			
Hispanic ethnicity	4.83	0.11–216.20	0.417			
≥4 Effective medications	3.46	0.41–29.47	0.256			
Age 45–64 y	3.41	0.39–30.21	0.271			
Asian race	3.35	0.07–151.32	0.534			
Disseminated TB disease	2.28	0.21–24.20	0.495			
Foreign born	2.10	0.15–28.64	0.577			
Recent cigarette smoker	1.96	0.41–9.31	0.397			
Recent excess alcohol use	0.99	0.13–7.65	0.988			
TB clinic outpatient management	0.98	0.18–5.35	0.980			
Recent unemployment	0.97	0.24–3.93	0.964			
Cavitary disease	0.77	0.21–2.84	0.694			
Diabetes	0.76	0.16–3.70	0.732			
History of TB disease	0.44	0.12–1.59	0.208			
HIV infection	0.39	0.03–4.92	0.463			
Recent injection drug or noninjection drug use	0.15	0.00–5.08	0.291			

*MDR TB, multidrug-resistant tuberculosis; XDR TB, extensively drug-resistant TB; OR, odds ratio; AFB, acid-fast bacilli; Pr, probability. Boldface indicates significance in the final model. Blank cells indicate variables not retained in the final model.
†Model fit intercept and covariates: Akaike information criterion, initial 136.392, final 116.619; Schwarz criterion, initial 198.964, final 127.996; –2logL, initial 92.392, final 108.619.

Cost of MDR TB and XDR TB

Direct costs averaged $134,000 (SE $9,683) per MDR TB patient and $430,000 (SE $73,109) per XDR TB patient. In comparison, costs are estimated at $17,000 (SE $1,210) per non-MDR TB patient (Figure 2 and online Technical Appendix). For isoniazid/rifampin/rifabutin-only, direct costs averaged $77,000 (SE $15,448) and direct-plus-productivity-loss costs averaged $226,000 (SE $73,338). Outpatient medications comprised ≈40% of direct costs, averaging $53,300 for MDR TB and $164,000 for XDR TB patients. Direct-plus-productivity-loss costs averaged $260,000 (SE $23,212) per MDR TB patient and $554,000 (SE = $127,707) per XDR TB patient. Highest costs were nearly $1.8 million. Applying these averages to 364 cases of MDR TB and 9 cases of XDR TB in the United States during 2005–2007, direct costs were ≈$53 million and direct-plus-productivity-loss costs were ≈$100 million.

When days hospitalized were controlled for, characteristics associated with greater direct costs, in descending order, were having XDR TB, residency in a long-term-care institution, non–injection-drug use, HIV infection, or having public insurance (adjusted $R^2 = 0.55$). When days hospitalized were controlled for, characteristics associated with greater direct-plus-productivity-loss costs were death, XDR TB, non–injection-drug use, HIV-infection, diabetes, or being male ($R^2 = 0.66$) (online Technical Appendix)

Table 2 shows that known insurance status was indicated on clinic records of 112 patients. Of 76 patients for whom insurance coverage while hospitalized was known, 38% had public insurance (including 32% Medicaid), 36% had no insurance, 24% had private insurance, 4% were in jail/prison, and 1% had other insurance. The public sector covered (i.e., by public insurance, for outpatients who had no/unknown insurance, for uninsured inpatients cared for in publicly financed hospitals, for jail/prison inmates) 75% of MDR TB and 100% of XDR TB patients and incurred 80% of MDR TB direct costs ($13,883,000/$17,415,000) and 100% of XDR TB direct costs ($2,149,000).

Discussion

In this population-based sample, which comprised 36% (130/364) of MDR TB and 56% (5/9) of XDR TB cases reported in the United States during 2005–2007, MDR/XDR TB diagnosis and treatment were very complex: *M. tuberculosis* isolates were resistant to a large number of medications, care was complicated by extensive disease and by concurrent conditions, and patients were highly infectious.

Despite this complexity, for nearly all eligible patients, sputum cultures converted to negative and 78% of patients completed treatment, including all those with XDR TB. Only 1% stopped treatment because of adverse events. The mortality rate (10%) was lower than that for other countries (*13–17*), and the mortality rate for patients during treatment (9%) was similar to that for US patients with isoniazid/rifampin-susceptible TB (8%) (L. Armstrong, pers. comm.). Among patients who died, 75% (9/12) of deaths

Table 5. Characteristics associated with expert consultation for 134 patients during MDR TB treatment, California, Texas, and New York, NY, USA, 2005–2007*†

Variable	Initial OR estimate	Initial 95% CI	Initial Pr>χ^2	Final OR estimate	Final 95% CI	Final Pr>χ^2
XDR TB	>999.999	<0.001–>999.999	0.939			
Recent homelessness	>999.999	<0.001–>999.999	0.887			
Correctional institution residence	>999.999	<0.001–>999.999	0.939			
Recent injection drug or noninjection drug use	61.63	0.00–>999.999	0.401			
Recent cigarette smoker	27.88	0.56–>999.999	0.096			
Diabetes	26.44	1.28–545.80	0.034			
Disseminated TB disease	9.49	0.18–501.11	0.266			
TB clinic outpatient management	7.96	1.50–42.32	0.015	**5.67**	**1.93–16.64**	**0.002**
Age 45–64 y	4.30	0.38–48.55	0.239			
Recent unemployment	3.81	0.52–28.18	0.190			
Age 25–44 years	2.92	0.58–14.63	0.192			
Long-term care facility resident	1.87	<0.001–>999.999	0.878			
5-drug regimen	1.13	0.22–5.82	0.882			
Total no. adverse events	1.12	0.88–1.42	0.371			
Total first-line resistance	0.63	0.11–3.77	0.614			
Acquired resistance	0.57	0.07–4.37	0.588			
Foreign born	0.55	0.03–12.28	0.705			
History of TB disease	0.53	0.13–2.10	0.363			
Age ≥65 y	0.50	0.02–14.97	0.687			
HIV infection	0.34	0.02–7.21	0.492			
Pregnant	0.28	0.02–5.15	0.393			
Private insurance	0.14	0.02–0.87	0.035	**0.23**	**0.08–0.68**	**0.008**
Pre-XDR	0.12	0.01–1.35	0.087			
Male	0.12	0.02–0.82	0.031			
Died	0.05	<0.001–3.46	0.165			
White race	0.05	<0.001–>999.999	0.988			
Recent excess alcohol use	<0.001	<0.001–0.06	0.002	**0.19**	**0.05–0.72**	**0.014**
Black race	<0.001	<0.001–>999.999	0.937			
Hispanic ethnicity	<0.001	<0.001–>999.999	0.938			
Asian race	<0.001	<0.001–>999.999	0.943			

*MDR TB, multidrug-resistant tuberculosis; XDR TB, extensively drug-resistant TB; OR, odds ratio. Boldface indicates significance in the final model. Blank cells indicate variables not retained in the final model.
†Model fit intercept and covariates: AIC (initial 133.402, final 121.683), SC (initial 223.235, final 133.274**); –**2logL (initial 71.402, final 113.683).

were TB-related, and 67% (6/9) occurred within 49 days of TB diagnosis. Among HIV-infected patients, failure or inability to use an effective injectable TB medication was associated with TB-related death.

Some diagnostic and treatment practices contributed to successful outcomes. Among symptomatic patients, initial TB diagnosis was made relatively quickly, within 7 weeks of symptom onset. Nearly three-fourths (73%) of patients were hospitalized; duration was 1 month for non-XDR TB patients and 9 months for XDR TB patients. Extensive DST of first-line and second-line medications was conducted within 2 months of treatment initiation for most patients. At some point during treatment, 61% of patients were receiving a 5-drug regimen. For outpatient care, DOT was used nearly universally, including during home isolation as recommended by national guidelines (*7*). The physicians of most patients with MDR/XDR TB consulted with experts. However, patient management required intensive monitoring and numerous medication changes. Case management services were also intensive; a case manager was assigned to ≈90% of patients.

Deficiencies in practices were identified. Despite CDC recommendations, ≈20% of patients had no documentation of expert consultation. Outcome was unknown for 3% who transferred within and 6% outside the United States and for 2% who were lost to follow-up. Acquisition of any drug resistance during treatment occurred for 21% of patients and was more likely to occur in populations difficult to treat (those who had pre-XDR TB or XDR TB or were homeless), suggesting a need for more vigilant treatment monitoring. There were delays of ≈3 months before patients started a 5-drug regimen. Today, the use of more rapid molecular diagnostic techniques could shorten the time to initiation of an appropriate treatment regimen (*18*). Since September 2009, CDC has offered US sites a molecular-based testing service (www.cdc.gov/tb/topic/laboratory/guide.html).

Because MDR TB treatment lasts >2 years (vs. 6 months for drug-susceptible TB), uses expensive medications, and requires hospitalization for ≈75% of patients (vs. 50% with drug-susceptible TB), it was very costly to treat and manage; average direct cost was $134,000 per MDR TB patient and $430,000 per XDR TB patient. The estimated $17,000 per non-MDR TB patient is 8 and 25 times lower than the costs for MDR TB and XDR TB, respectively. In contrast, lifetime care per HIV-infected

Table 6. Treatment outcomes of MDR/XDR TB study patients alive at diagnosis, by resistance pattern, California, Texas, and New York, NY, USA, 2005–2007*

Resistance pattern	Completed treatment, %	Transferred within United States, %	Transferred out of United States, %	Lost to follow-up, %	Stopped because of side effects, %	Died during treatment, %
All, N = 134	78	3	6	2	1	9
INH/RIF/RBT-only, n = 17	59	0	18	6	0	18
INH/RIF/RBT-plus, n = 58	83	3	7	0	0	7
First-line, n = 32	78	0	3	6	3	9
Pre-XDR, n = 22	77	9	0	0	5	9
XDR, n = 5	100	0	0	0	0	0

*MDR TB, multidrug-resistant tuberculosis; XDR TB, extensively drug-resistant TB; INH, isoniazid; RIF, rifampin; RBT, rifabutin.

patient costs $380,000 (updated to 2010 dollars) (*19*) and lifetime care per breast cancer patient costs $20,000–$100,000 (*20*).

During 2005–2007, the 373 MDR/XDR TB cases cost the US health care system an estimated $53 million; during this time there were ≈41,000 total TB cases. Direct costs for an average XDR TB patient were 3.2 times those for an average MDR TB patient, mostly because of hospitalization costs. Only 20% of XDR TB and 28% of MDR TB patients were managed solely as outpatients. The public sector incurred 80% of the MDR TB costs and 100% of the XDR TB costs.

With health care reform, a substantial proportion of uninsured TB patients are expected to become eligible for Medicaid coverage, which should increase access to health care and early TB diagnosis and decrease TB-associated hospitalizations and deaths. Prevention opportunities for MDR TB are limited. Maintaining the capacity of public health departments and of publicly financed hospitals to act as safety nets (regardless of patient insurance status) to quickly diagnose MDR/XDR TB and isolate and effectively treat the patients will be critical for preventing deaths and transmission of drug-resistant TB organisms. Investment in infection control infrastructure and the capacity to prevent TB among MDR TB patient contacts is also critical; our cost-of-illness estimates did not include these programmatic costs of preventing cases. During the 1979–1994 TB resurgence and MDR TB outbreaks in the United States, New York City renovated hospitals and the Rikers Island prison and treated 20,000 excess TB patients at a cost of ≈$1.7 billion (updated to 2010 dollars) (*21*).

This study had some limitations. Detailed hospitalization records were unavailable for 17 patients. Moreover, documentation of care for incarcerated patients was limited. Follow-up data for all patients were unavailable after 1 year of treatment. Because outpatient care was provided by a mixture of public and private providers, we used average wholesale medication prices to estimate medication costs, which overestimated actual costs to TB clinics that have access to reduced (often one half to one third) medication prices negotiated with pharmaceutical companies. Estimates of out-of-pocket costs were not included.

Conclusions

In this population-based sample of MDR/XDR TB patients in the United States, despite the extensive drug resistance found at the time of diagnosis, culture conversion and treatment completion rates were high and mortality rates were low. Records of treatment practices documented near-universal use of DOT. However, these outcomes came at a high cost to the public sector, providing incentives for

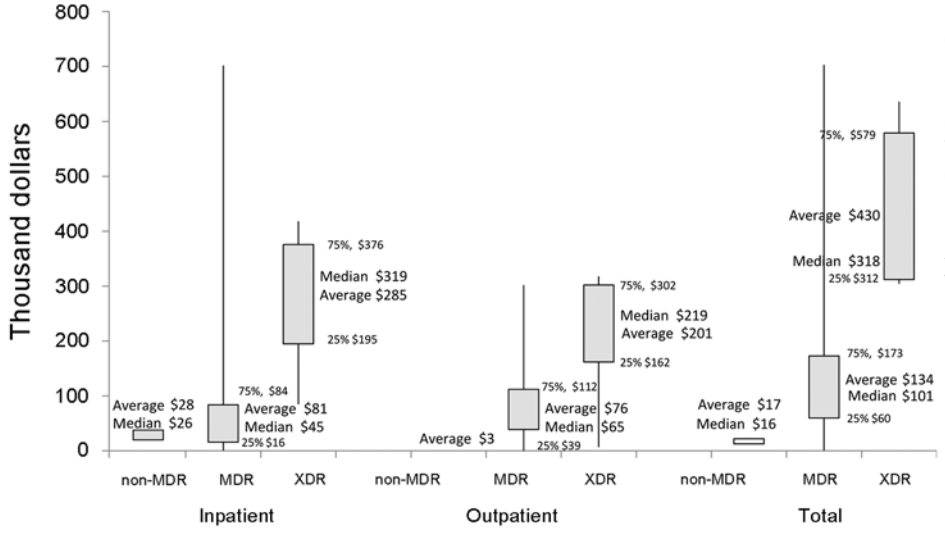


Figure 2. Average, median, and distribution of direct costs per patient in 2010 US dollars by drug resistance. This box-plot diagram shows the minimum and maximum values (vertical lines), the averages and medians (numbers), and the interquartile ranges (box). MDR, multidrug-resistant tuberculosis; XDR, extensively drug-resistant tuberculosis.

the United States to prevent MDR/XDR TB. Preventing MDR/XDR TB in the United States will require addressing factors associated with development of drug resistance in countries where foreign-born US patients originate, as well as rapid diagnosis, appropriate regimen selection, robust case management practices, and continued emphasis on DOT in the United States.

Acknowledgments

We acknowledge the efforts of many persons involved with study data collection and management and data analysis consultation, including Holly Anger, Pennan Barry, Peter Cegielski, Jeffrey Chrismon, Carla Cueva, Wafaa El-Sadr, Denise Garrett, Jerrie Givens, Anita Musafar, Thomas Navin, Hugo Ortega, Stephanie Ott, Nicolette Palermo, Vicki Randle, Alicia Rodriguez, Marthe Sende, Brian Sizemore, John Stamper, Andrew Vernon, Charles Wallace, Ying Wang, and James Watt.

This work was supported by the Tuberculosis Epidemiologic Studies Consortium of CDC. The CDC National Center for HIV, Viral Hepatitis, STD and TB Prevention and the Division of Tuberculosis Elimination supported CDC and contract staff and the analytical resources used during the project.

Ms Marks is an epidemiologist/economist in the Division of Tuberculosis Elimination at CDC. Her research interests include combining epidemiologic research and economic analysis of TB diagnostics, MDR TB treatment, outcomes, and prevention, and often focus on populations experiencing health disparities, such as persons living with HIV or homelessness, and sexual/gender minorities.

References

1. Munsiff SS, Ahuja SD, Li J, Driver CR. Public–private collaboration for multidrug-resistant tuberculosis control in New York City. Int J Tuberc Lung Dis. 2006;10:639–48.
2. Burgos M, Gonzalez LC, Paz EA, Gournis E, Kawamura LM, Schecter G, et al. Treatment of multidrug-resistant tuberculosis in San Francisco: an outpatient-based approach. Clin Infect Dis. 2005;40:968–75. http://dx.doi.org/10.1086/428582
3. Shah NS, Pratt R, Armstrong L, Robison V, Castro KG, Cegielski JP. Extensively drug-resistant tuberculosis in the United States, 1993–2007. JAMA. 2008;300:2153–60. http://dx.doi.org/10.1001/jama.300.18.2153
4. Banerjee R, Allen J, Westenhouse J, Oh P, Elms W, Desmond E, et al. Extensively drug-resistant tuberculosis in California, 1993–2006. Clin Infect Dis. 2008;47:450–7. http://dx.doi.org/10.1086/590009
5. Rajbhandary SS, Marks SM, Bock NN. Costs of patients hospitalized for multidrug-resistant tuberculosis. Int J Tuberc Lung Dis. 2004;8:1012–6.
6. Althomsons SP, Cegielski JP. Impact of second-line drug resistance on tuberculosis treatment outcomes in the United States: MDR TB is bad enough. Int J Tuberc Lung Dis. 2012;16:1331–4. http://dx.doi.org/10.5588/ijtld.11.0812
7. Centers for Disease Control and Prevention. Treatment of tuberculosis. American Thoracic Society, CDC, and Infectious Diseases Society of America. MMWR Recomm Rep. 2003;52(RR-11):1–80.
8. Falzon D, Jaramillo E, Schünemann HJ, Arentz M, Bauer M, Bayona J, et al. WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. Eur Respir J. 2011;38:516–28. http://dx.doi.org/10.1183/09031936.00073611
9. Centers for Medicare & Medicaid Services. Operating cost to charge ratios (OPCCR). FY2005 Impact file (inpatient prospective payment system) 2005 [cited 2010 May 12]. http://www.cms.gov/Medicare/Medicare-Fee-for-Service-Payment/PCPricer/index.html?redirect=/pcpricer
10. Bureau of Labor Statistics. Consumer Price Index—all urban consumers, medical care. Series ID CUUR0000SAM [cited 2012 Feb 21]. http://data.bls.gov/cgi-bin/srgate
11. Centers for Medicare & Medicaid Services. Medicare geographic adjustment factors [cited 2010 May 12]. http://www.cms.gov/Research-Statistics-Data-and-Systems/Statistics-Trends-and-Reports/Medicare-Geographic-Variation/GV_PUF.html
12. Grosse SD. Appendix I: productivity loss tables. In: Haddix AC, Teutsch SM, Corso PS, editors. Prevention effectiveness: a guide to decision analysis and economic evaluation. 2nd ed. New York: Oxford University Press; 2003. p. 245–57.
13. Johnston JC, Shahidi NC, Sadatsafavi M, Fitzgerald JM. Treatment outcomes of multidrug-resistant tuberculosis: a systematic review and meta-analysis. PLoS ONE. 2009;4:e6914. http://dx.doi.org/10.1371/journal.pone.0006914
14. Orenstein EW, Basu S, Shah NS, Andrews JR, Friedland GH, Moll AP, et al. Treatment outcomes among patients with multidrug-resistant tuberculosis: systematic review and meta-analysis. Lancet Infect Dis. 2009;9:153–61. http://dx.doi.org/10.1016/S1473-3099(09)70041-6
15. Jacobson KR, Tierney DB, Jeon CY, Mitnick CD, Murray MB. Treatment outcomes among patients with extensively drug-resistant tuberculosis: systematic review and meta-analysis. Clin Infect Dis. 2010;51:6–14. http://dx.doi.org/10.1086/653115
16. Ahuja SD, Ashkin D, Avendano M, Banerjee R, Bauer M, Bayona JN, et al. Multidrug resistant pulmonary tuberculosis treatment regimens and patient outcomes: an individual patient data meta-analysis of 9,153 patients. PLoS Med. 2012;9: e1001300. http://dx.doi.org/10.1371/annotation/230240bc-bcf3-46b2-9b21-2e6e584f7333
17. Dalton T, Cegielski P, Akksilp S, Asencios L, Campos Caoili J, Cho SN, et al. Prevalence of and risk factors for resistance to second-line drugs in people with multidrug-resistant tuberculosis in eight countries: a prospective cohort study. Lancet. 2012;380:1406–17. http://dx.doi.org 10.1016/S0140-6736(12)60734-X
18. Banerjee R, Allen J, Lin SYG, Westenhouse J, Desmond E, Schecter GF, et al. Rapid drug susceptibility testing with a molecular beacon assay is associated with earlier diagnosis and treatment of multidrug-resistant tuberculosis in California. J Clin Microbiol. 2010;48:3779–81. http://dx.doi.org/10.1128/JCM.01236-10
19. Hutchinson AB, Farnham PG, Duffy N, Wolitski RJ, Sansom SL, Dooley SW, et al. Return on public health investment: CDC's expanded HIV testing initiative. J Acquir Immune Defic Syndr. 2012;59:281-6. PMID: 22067662 doi: 10.1097/QAI.0b013e31823e5bee
20. Campbell JD, Ramsey SD. The costs of treating breast cancer in the U.S.: a synthesis of published evidence. Pharmacoeconomics. 2009;27:199–209. http://dx.doi.org/10.2165/00019053-200927030-00003
21. Frieden TR, Fujiwara PI, Washko RM, Hamburg MA. Tuberculosis in New York City—turning the tide. N Engl J Med. 1995;333:229–33.

Address for correspondence: Suzanne M. Marks, Centers for Disease Control and Prevention, Mailstop E10, 1600 Clifton Rd NE, Atlanta, GA 30333, USA; email: smarks@cdc.gov

etymologia

Papillomavirus

[pap-ĭ-lo-mə-vi-rəs]

From the Latin *papillo-* ("nipple") + *oma* ("tumor"), papillomaviruses are nonenveloped DNA viruses that induce exophytic lesions of the skin and mucous membranes. The first animal papillomavirus was described in 1933 by Richard Shope, who researched papillomata in "warty" wild cottontail rabbits. In 1975, Harald zur Hausen published the hypothesis that the human papillomavirus played a role in the etiology of cervical cancer, work for which he was awarded the Nobel Prize in Physiology or Medicine in 2008.

Sources

1. Howley PM, Schiller JT, Lowy DR. Papillomaviruses. In: Knipe DM, Howley PM, editors. Fields virology. 6th ed. Philadelphia: Lippincott Williams and Wilkins; 2013. p. 1662–703.
2. Shope RE, Hurst EW. Infectious papillomatosis of rabbits. J Exp Med. 1933;58:607–24. http://dx.doi.org/10.1084/jem.58.5.607
3. zur Hausen H, Gissman L, Steiner W, Dippold W, Dreger I. Human papilloma viruses and cancer. Bibl Haematol. 1975;(58):569–71.

Address for correspondence: Ronnie Henry, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E03, Atlanta, GA 30333, USA; email: boq3@cdc.gov

DOI: http://dx.doi.org/10.3201/eid2005.ET2005

Human Papillomavirus Prevalence in Oropharyngeal Cancer before Vaccine Introduction, United States

Martin Steinau, Mona Saraiya, Marc T. Goodman, Edward S. Peters, Meg Watson, Jennifer L. Cleveland, Charles F. Lynch, Edward J. Wilkinson, Brenda Y. Hernandez, Glen Copeland, Maria S. Saber, Claudia Hopenhayn, Youjie Huang, Wendy Cozen, Christopher Lyu, Elizabeth R. Unger, and the HPV Typing of Cancers Workgroup[1]

We conducted a study to determine prevalence of HPV types in oropharyngeal cancers in the United States and establish a prevaccine baseline for monitoring the impact of vaccination. HPV DNA was extracted from tumor tissue samples from patients in whom cancer was diagnosed during 1995–2005. The samples were obtained from cancer registries and Residual Tissue Repository Program sites in the United States. HPV was detected and typed by using PCR reverse line blot assays. Among 557 invasive oropharyngeal squamous cell carcinomas, 72% were positive for HPV and 62% for vaccine types HPV16 or 18. Prevalence of HPV-16/18 was lower in women (53%) than in men (66%), and lower in non-Hispanic Black patients (31%) than in other racial/ethnic groups (68%–80%). Results indicate that vaccines could prevent most oropharyngeal cancers in the United States, but their effect may vary by demographic variables.

Oropharyngeal cancers include malignancies that occur where the oral cavity and pharynx merge, including in the palatine and lingual tonsils, the posterior 1/3 (base) of the tongue, the soft palate, and the posterior pharyngeal wall. Current worldwide incidence has been estimated at ≈85,000 annually (*1*), although it varies extensively by geographic region. In the United States, ≈12,000 new oropharyngeal cancers are diagnosed annually (*2*); most are classified histologically as squamous cell carcinoma (OPSCC). In addition to tobacco use and alcohol use, infection with human papillomavirus (HPV) has been recognized as an independent risk factor for oropharyngeal cancer (*3–6*).

The 2 HPV vaccines approved by the US Food and Drug Administration protect against infection with HPV-16 and HPV-18, which are the high-risk types most frequently associated with cervical cancer. A candidate 9-valent vaccine that includes types in the existing quadrivalent vaccine (HPV types 6, 11, 16, and 18) and 5 additional high-risk types (31, 33, 45, 52, and 58) is in clinical trial. Supported by evidence that existing vaccines effectively reduce oral HPV infections, these formulations may also reduce incidence of oropharyngeal cancers (*7*). When monitoring the population-level effect of HPV vaccination on oropharyngeal cancer occurrence in the United States, data on the incidence and type-specific prevalence of this disease are essential. Previously, the prevalence of cases attributable to viral infection and the consequent effects of vaccine programs were approximated from small published studies (*8–13*), which estimated HPV to be detected in 37%–60% of OPSCC in North America. Considering the range of prevalence, the heterogeneity of study populations, and differences in sample preparation and HPV detection methods used in these studies, it is not clear that this range of estimates reflects the true scope of HPV-associated OPSCC in the United States. Therefore, the objectives of this study were to determine prevalence of HPV types detected in oropharyngeal cancers in the United States and to establish a prevaccine baseline for monitoring the impact of vaccination.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (M. Steinau, M. Saraiya, M. Watson, J.L. Cleveland, E.R. Unger); Cedars-Sinai Medical Center, Los Angeles, California, USA (M.T. Goodman); Louisiana State University, New Orleans, Louisiana, USA (E.S. Peters); University of Iowa, Iowa City, Iowa, USA (C.F. Lynch); University of Florida, Gainesville, Florida, USA (E.J. Wilkinson); University of Hawaii, Honolulu, Hawaii, USA (B.Y. Hernandez); Michigan Cancer Surveillance Program, Lansing, Michigan, USA (G. Copeland); Los Angeles Cancer Registry, Los Angeles, California, USA (M.S. Saber, W. Cozen); University of Kentucky, Lexington, Kentucky, USA (C. Hopenhayn); Florida Department of Health, Tallahassee, Florida, USA (Y. Huang); and Battelle Memorial Institute, Durham, North Carolina, USA (C. Lyu)

DOI: http://dx.doi.org/10.3201/eid2005.131311

[1]Members of HPV Typing of Cancers Workgroup who contributed to this study are listed at the end of this article.

Materials and Methods

Cancer Tissue Specimens

As part of the Centers for Disease Control Cancer Registry Sentinel Surveillance System study (M. Saraiya, unpub data), a systematic review of cases of oropharyngeal cancer diagnosed during 1995–2005 was performed. The cases were selected from 7 participating registries, including 4 central cancer registries in Florida, Kentucky, Louisiana, and Michigan and 3 Surveillance, Epidemiology, and End Results program (SEER [http://seer.cancer.gov/]) cancer registry-based residual tissue repositories in Los Angeles County, CA; Hawaii; and Iowa. The following anatomic regions (by ICD-O-3 codes) were included: C01.9 and C02.4 (base of the tongue and lingual tonsil); C09.0, C09.1, C09.8, C09.9, and c14.2 (tonsil); C14.0, C14.2, C14.8, C02.8, C10.2, C10.8, and C10.9 (other oropharynx) (*13*). Of 4,073 cases matching these criteria, we requested samples from 1,271 case-patients representative of the whole case-patient population regarding sex, age, and race/ethnicity. One archived, formalin–fixed paraffin-embedded tissue sample, representative of the primary tumor, was selected by the submitting pathology laboratory. If tissue from the primary tumor was unavailable, a sample from a metastatic lesion in a lymph node was accepted because HPV prevalence is usually maintained in OPSCC–positive lymph nodes (*14*). With the exception of 32 cases from Hawaii and 11 from Los Angeles County, which had been sampled by Chaturvedi et al. (*12*), cases were selected exclusively for this study. Each participating state and CDC received approval from their institutional review boards for the study; CDC approved the overall study.

DNA Extraction and HPV Typing

All laboratory methods were described previously (*15*,*16*). Six consecutive 5-μm sections were cut from each selected tissue block; special precautions were used to avoid cross-contamination. The first and last sections were stained with hematoxylin and eosin and reviewed by a study pathologist (ERU) to confirm the presence of viable tumor tissue. DNA was extracted from two 5-μm sections by using high temperature–assisted tissue lysis (*17*) and further purification was carried out by automated extraction by using Chemagic MSM1 (PerkinElmer, Waltham, MA, USA). HPV types were determined from 2 commercial assays by using an algorithm which was evaluated earlier for this application (*18*,*19*). First, all DNA extracts were tested by using the Linear Array HPV Genotyping Assay (Linear Array; Roche Diagnostics, Indianapolis, IN, USA) and a HPV-52-specific PCR to resolve ambiguous positive results from the XR probe of the Linear Array HPV test (*20*). Samples that had negative or inadequate linear array results (negative for HPV and cellular β-globin controls) were retested with the INNO-LiPA HPV Genotyping Assay (Innogenetics, Gent, Belgium). HPV status was recorded for 40 types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, IS39, and 89 as tested; and HPV of an unknown type (HPV-X) for additional unspecified types as indicated by LiPA results.

Analysis

Prevalence was assessed as percentage positive from the total number of cases with valid results. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were considered to have a high risk for oncogenic potential (*21*) and all other types, including HPV-X, to have a low risk, showing low or no known oncogenic potential.

Hierarchical categories for HPV status were assigned as follows: HPV-16 includes all cases positive for this type regardless of other results. HPV-18 includes all cases positive for HPV-18, but not for HPV-16; other 9-valent, high-risk HPV types included in the next generation of the HPV vaccine: HPV-31, -33, -45, -52, -58, but not HPV-16 or -18; other high-risk HPV cases positive for any high risk not included in the previous categories: HPV-35, -39, -51, -66, -68; and low-risk HPV: all other cases positive for any remaining low-risk HPV types.

Statistical analysis was restricted to case-patients that had confirmed invasive OPSCC. Case-patient age at diagnosis was stratified into 4 groups: <50, 50– 59, 60–69, and ≥70 years. Cancer stages were crudely classified as local, regional, or distant (metastatic) by SEER classifications. Differences in prevalence of positive results for high-risk HPV or HPV-16/18, categorized by patient's age, sex, race/ethnicity, and the anatomic location of cancer, were evaluated by using the χ^2 or Fisher exact test whenever possible. Multivariate analysis was performed by using logistic regression with a step-down procedure, adjusting for age, sex, and race/ethnicity as appropriate. All statistical calculations were made by using SAS 9.3 (SAS Institute Inc., Cary, NC, USA).

Results

Of the 1,271 oropharyngeal tumors requested from the participating cancer registries, samples from 588 case-patients were received and successfully tested. Those not received were either unavailable or the remaining tissue was not representative of disease. The demographic characteristics (sex, age) and cancer stage (Table 1) (*22*) of the cohort from which the tested sample set was collected were similar to those of the untested cohort. Persons from the Asian Pacific Islands were few in number and slightly overrepresented in the final test population.

HPV results for 476 (81.0%) samples were from the linear array and 112 (19%) from LiPA. Most tissue was

Table 1. Characteristics of oropharyngeal cancer case-patients who provided samples compared with those of all eligible case-patients, United States, 1995–2005*

Characteristic	% Case-patient samples not tested, n = 683	% Case-patient samples tested, n = 588
Age, y		
<50	18.4	18.5
50–59	32.1	33.7
60–69	27.5	27.6
≥70	22.0	20.2
Race/ethnicity		
Asian/Pacific Islander	1.3	4.4
Non-Hispanic Black	11.9	12.6
Hispanic	8.6	6.8
Non-Hispanic White	77.6	75.2
Other	0.6	1.0
Sex		
F	23.4	25.4
M	76.6	74.5
Cancer stage		
Local	13.6	19.2
Regional	52.0	56.5
Distant	17.7	13.6
Unknown	16.7	10.7

*Samples were provided from 7 US registries.

from the primary site (n = 473), but for 15 samples, only metastatic tissue from lymph nodes was available. Most case-patients (77.6%) were from urban areas or counties with a population >250,000. Most (94.4%) diagnoses were made during 2000 or later. Median age at the time of diagnosis was 58 (range 28–97) years. The male-to-female ratio was 3:1 and most of the cases (75.6%) were in non-Hispanic White persons. SCC, the most common histologic type of oropharyngeal cancer, accounted for 557 (94.7%) of all cases, and the main analysis was restricted to these cases (Table 2).

HPV was detected in 403 of the 557 OPSCC cases (72.4%) with valid typing results and 396 (71.1%) were positive for ≥1 high-risk type (Table 3). In 68.4% of cases, a single HPV type was found; 3.9% contained 2 types. In 7 cases, only low-risk HPV types were detected: HPV-11, 26, 69, 82 (2 cases), 83, and HPV-X). HPV-16 was present in 337 (60.5%) cases, HPV-18 in 14 (2.5%) cases, and 331 (59.4%) cases were exclusively positive for these 2 types.

Other high-risk types, including HPV-31, -33, -35, -39, -45, and -52, were found at low frequency (Table 3). The relative prevalence in case-patients that had multiple HPV types essentially followed single–type distributions. HPV-16/33 was the most frequent combination (6 cases); HPV-16/18 and HPV-16/31 were the next most frequent, found in samples from 3 case-patients each. Frequencies of all co-detected HPV types are shown in Table 4. More than 2 types were not found in any of the oropharyngeal cancers.

Proportions of high-risk HPV prevalence and HPV-16/18 were statistically different among the registries and by race/ethnicity, stage, and anatomic subsite (Table 2). By sex, prevalence was only different for those infected with HPV-16/18. Age at diagnosis was not statistically different between the stratified groups, but median age at diagnosis among high-risk HPV positive case-patients was 58 (28–92) years and 61 (36–97) years in high-risk negative case-patients (p = 0.023).

According to hierarchical assessment, HPV-16 was found in 337 (60.5%) OPSCC cases, HPV-18 in 11 (2.0%), other 9-valent high-risk types in 32 (5.7%), other high-risk types in 16 (2.9%), and low-risk types in the remaining 7 (1.3%) cases (Figure 1). Of the 15 case-patients for whom lymph node metastases were tested, 14 were positive for high-risk HPV and 13 were positive for HPV-16.

In multivariate analysis for high-risk HPV that included age and sex in the model, only race/ethnicity was a significant independent factor (p = 0.003). Odds for high-risk

Table 2. High-risk and HPV types16 and 18 in oropharyngeal squamous cell carcinomas by demographic and tumor characteristics, select United States registries, 1995–2005*

Characteristic	Total no.	High-risk HPV, no. (%) pos.†	HPV-16/18, no. (%) pos.‡
Registry			
Los Angeles Co., CA	20	17 (85.0)	14 (70.0)
Florida	140	101 (72.1)	89 (63.6)
Hawaii	39	33 (84.6)	32 (82.1)
Iowa	13	4 (30.7)	4 (30.7)
Kentucky	116	74 (63.8)	69 (59.5)
Louisiana	95	75 (78.9)	61 (64.2)
Michigan	134	92 (68.6)	79 (59.0)
p value	NA	<0.001	0.032
Age, y			
<50	106	83 (78.3)	74 (69.8)
50–59	191	142 (74.3)	127 (66.5)
60–69	156	102 (65.4)	89 (57.1)
≥70	104	69 (65.4)	58 (55.8)
p value	NA	0.064	0.053
Sex			
F	141	91 (64.5)	74 (52.5)
M	416	305 (74.3)	274 (65.9)
p value	NA	0.053	0.006
Race/ethnicity			
Asian/Pacific Islander	20	16 (80.0)	16 (80.0)
Non-Hispanic Black	71	36 (50.7)	22 (31.0)
Hispanic	39	29 (74.4)	27 (69.2)
Non-Hispanic White	421	310 (73.6)	278 (67.5)
Other	6	5 (83.3)	5 (83.3)
p value	NA	0.002	<0.001
Tumor stage			
Local	102	60 (58.8)	51 (50.0)
Regional	318	248 (78.0)	225 (70.8)
Distant (metastatic)	76	51 (67.1)	42 (55.3)
Unknown	61	37 (60.7)	30 (49.2)
p value	NA	0.001	<0.001
Tumor subsite			
Base of tongue	213	149 (70.0)	129 (60.6)
Tonsil	250	201 (80.4)	181 (72.4)
Other	94	46 (48.9)	38 (40.4)
p value	NA	<0.001	<0.001

*p values for differences between categories were calculated by χ^2/Fisher exact test. HPV, human papilloma virus; pos., positive; Co., county; NA, not applicable.
†Positive for any of the high-risk types: HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68.
‡HPV-16/18 = positive for HPV-16 and/or HPV-18.

Table 3. Human papillomavirus prevalence in oropharyngeal squamous cell carcinomas, select United States registries, 1995–2005*

Variable	No. (%) cases, N = 557
Characteristic	
HPV (any type)	403 (72.4)
High risk†	396 (71.1)
Low risk‡	7 (1.3)
Negative	154 (27.6)
Single type	381 (68.4)
Multiple types§	22 (3.9)
Type	
HPV-16	337 (60.5)
HPV-33	31 (5.6)
HPV-18	14 (2.5)
HPV-35	11 (2.0)
HPV-39	5 (0.9)
HPV-31	4 (0.7)
HPV-52	4 (0.7)
HPV-45	3 (0.5)
Other HPV types	16 (2.9)

*HPV, human papillomavirus; high risk, virus type is associated with high oncogenic potential; low risk, virus type is associated with low oncogenic potential.
†Positive for any of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.
‡Positive for types other than those identified as high risk.
§HPV-16/-33 (6 cases), HPV-16/-18 (3 cases), and HPV-16/-31(3 cases).

HPV infections were significantly higher for all other race groups than for non-Hispanic Black persons ($p<0.001$). When only HPV-16/18 detection was considered, significant differences were found in sex ($p = 0.009$) and race/ethnicity in ($p<0.001$), but not age ($p = 0.063$), between those infected and those who were not (Table 5).

The 31 cases that had histologic results other than SSC included 7 adenocarcinomas (2 were HPV-16 positive, 5 HPV negative) and 2 small cell or neuroendocrine carcinomas (both HPV negative). Twenty-two cases were carcinomas not further specified, of which 7 tested positive for HPV (4 for HPV-16, and 1 each for HPV-18, HPV-33, HPV-35).

Discussion

Our finding of >70% HPV prevalence in a large sample of the oropharyngeal cancer patients from around the United States suggests that a substantially higher faction may be HPV-related than has been reported in many previous investigations (*14*). In a systematic review of HPV prevalence studies, including several small investigations of populations in North America, Kreimer et al. estimated that 47% of OPSCC cases were HPV related (*11*). Chaturvedi et al. more recently estimated weighted HPV prevalence at 72% (*12*), which is comparable to our findings. A continuous increase in HPV-related OPSCC that was observed during the past 20 years and escalated after 2000 (*23*) may explain some of the discrepancies found in the literature. More sensitive laboratory methods may also have contributed to the increased HPV prevalence relative to earlier investigations. All studies in North America considered by Kreimer et al. (*11*) relied on the MY09/11 or GP5+/6+ consensus primer sets that require intact HPV L1 fragments of 450 and 150 bp, respectively. By contrast, in the study by Chaturvedi et al. (*12*) and ours, testing incorporated the INNO-LiPA assay, which has SFP primers that target 65-bp amplicons. Assays targeting smaller L1 amplicons can achieve increased sensitivity for HPV detection in formalin-fixed, paraffin-embedded tissues known to have smaller DNA templates in their extracts than fresh or frozen samples do (*18*). Although not identical in specificity to that of the Linear Array, INNO-LiPA had shown comparable performance for detecting single type HPV infections predominantly found in cancer tissues (*19*).

Our current study results further confirm a trend of increasing incidence of tonsillar cancers with shifting demographic patterns (*24*,*25*).The results further confirmed that HPV-16, detected in 84% of all positive tissues, was by far the most frequent type found in oropharyngeal cancers. Although type 16 also has nominally the highest prevalence in the "normal" oral cavity or oropharynx, other types are usually found at similar frequency (*26*,*27*). The ability of HPV-16 to establish persistent infection and its potential to transform might be responsible for its prominence in cancers. Currently available HPV vaccines targeting HPV-16 and -18 may be highly effective against OPSCC (*9*). A candidate 9-valent vaccine (currently in clinical trials) could have the potential to prevent virtually all HPV-associated oropharyngeal cancers: our data showed that 2.9% of the case-patients were positive for a high-risk type not covered in this formulation. (Figure 1).

The most noticeable differences were observed between racial groups, with notably fewer HPV-positive SCCs in non-Hispanic Black persons (50.7%) compared with non-Hispanic White persons (73.6%), Hispanic persons (74.4%), or Asian Pacific Islanders (80.0%). Other studies that noted similar differences by race/ethnicity found this to be a recent but ongoing development (*28*). Settle et al. (*29*), who investigated oral cancer survival, also reported reduced HPV prevalence in Black persons

Table 4. Oropharyngeal squamous cell carcinomas with >1 HPV type, select US registries, 1995–2005*

Type combination	No. cases
HPV-16 and -33	6
HPV-16 and -18	3
HPV-16 and -31	3
HPV-16 and -35	2
HPV-16 and -45	1
HPV-16 and -52	1
HPV-16 and -54	1
HPV-16 and -59	1
HPV-16 and -83	1
HPV-18 and -35	1
HPV-33 and -39	1
HPV-39 and -56	1

*No cases with >2 types were found; HPV, human papillomavirus.

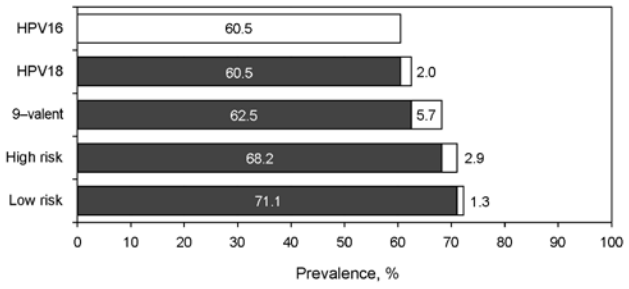


Figure. Hierarchical designation of human papillomavirus (HPV) types to oropharyngeal squamous cell carcinomas. White sections of bars indicate attribution of the specific HPV type or group. Black sections of bars indicate cumulative prevalence of types in higher hierarchy. HPV-16 includes all cases positive for this type regardless of other results. HPV-18 includes all cases positive for HPV-18, but negative for HPV-16. Cases of 9-valent HPV with high-risk HPV types included in the candidate 9-valent HPV vaccine: HPV-31, -33, -45, -52, -58, but not HPV-16 or -18. High-risk: cases positive for any high-risk type not included in the previous categories: HPV-35, -39, -51, -66, -68. Low-risk: cases only positive for HPV types with low or no oncogenic potential.

compared with other race/ethnicity groups and found that this difference was particular to oropharyngeal cancer and not to other cancers of the oral cavity.

In addition to differences by race/ethnicity, HPV prevalence also varied by sex, particularly for HPV-16/18. Prevalence was 66% among men, notably higher than the 53% found among women, which was a finding consistent with results of other investigations (*2*). Although further data stratification might show even greater dissimilarities, for instance between Black women and White men, the sample sizes for these analyses were modest and confidence intervals were large (data not shown). The precise causes for these discrepancies are unknown and most likely complex, but may be anticipated to influence vaccine efficacy for OPSCC. Prevalence differences observed between the registry states may be, in part, caused by demographic variations. Difference in age at diagnosis between patients with HPV-positive and HPV-negative cases was borderline significant ($p = 0.023$). Although other studies have also shown that HPV-positive cancers occur in a younger population (*30*), the role of 3 years difference in median age is unclear. It is possible that differences in behavior associated with causal pathways, such as smoking and drinking, provide a partial explanation. Persistent HPV infection at these anatomic sites may occur early, leading to more rapid and damaging alterations in cell cycle regulation and proliferation than those that occur with other carcinogenic exposures.

Of particular note, high-risk HPV types were detected in 80% of tonsillar SCCs. The microanatomy of the lymphoepithelial tissue of Waldeyer's ring, most notably the lingual and palatine tonsils, may explain this finding. Deep invaginations in this area by the tonsillar crypts may expose immature basal cells to HPV (*31*). The median age of case-patients was slightly lower than that of those with cancer in other sites (55 years), but proportions of infection, when sex and race/ethnicity were considered, were not different than proportions for the other oropharyngeal sites (data not shown). One limitation of this study is that not all participating sites were able to perform systematic random sampling of case-patient specimens from their eligible pool. The sizable specimen collections from the 4 cancer registries (Michigan, Kentucky, Louisiana, and Florida) were sampled by a simple random or systematic sampling approach, on the basis of the number of eligible cases. Sampling from the SEER tissue repositories (Los Angeles, California; Hawaii; and Iowa) was dependent on what tissue specimens were available. However, the resulting sample population that was ultimately tested represented diverse geographic regions and a wide range of demographic variables regarding sex, age, and race/ethnicity.

It should be noted that the presence of HPV DNA does not confirm its causal role in carcinogenesis. Detection in tumor tissues potentially overestimates the true involvement of the virus because coincidental, transient infections and complementary transforming effects to other factors cannot be distinguished. The natural history of cases in this study could not be assessed in this retrospective cross-sectional study and behavioral data were not available from the participating cancer registries. In particular, information regarding tobacco or alcohol use and HIV status would potentially improve estimation of the proportion of OPSCC caused by HPV alone. Because it is not clear at this point if HPV alone is sufficient to cause oropharyngeal cancer, factors other than use of tobacco products should be considered. Additional HPV markers, such as viral transcription (particularly E6 and E7 mRNA) or characteristic gene expression profiling, may provide further insights in future assessments and show distinction between actively transforming HPV infections and random, transient occurrences (*32*,*33*). Similar investigations may also be warranted to explicate the 7 cases

Table 5. Multivariate analysis for HPV and 18 detection in 557 oropharyngeal squamous cell carcinoma samples, select United States registries, 1995–2005*

Variable	OR (95% CI)†	p value
Sex		0.009
F	Ref	
M	1.70 (1.14–2.55)	
Race/ethnicity		<0.001
Non-Hispanic Black	Ref	–
Asian/Pacific Islander	8.43 (2.51–28.29)	–
Hispanic	4.73 (2.02–11.1)	–
Non-Hispanic White	4.34 (1.17–97.47)	–

*HPV, human papillomavirus; OR, odds ratio; Ref, reference group.
†Odds ratio adjusted for race and ethnicity; age was not significant.

in which only low-risk types were found. It is likely that these HPV types were present coincidentally and played no role in malignant transformation, but genomic changes that altered their pathogenic properties to bring them closer to those of high-risk types could provide an intriguing alternative explanation.

Conclusions

This study supports a role for oncogenic HPV in high proportions of oropharyngeal cancers. Future assessments are needed to monitor general prevalence and possible type-specific shifts. Data from the present and future studies will provide a baseline for early assessment of vaccine effects. Because the natural history and pre-cancer stages of oropharyngeal cancers are not established as they are for cervical cancer, direct trials with oropharyngeal neoplasia as the endpoint are not feasible. To obtain meaningful, comparable data for this objective, researchers need a universal definition of the anatomic oropharynx and associated malignancies and agreement on laboratory methods.

Members of the HPV Typing of Cancers Workgroup are the following: Battelle (Bruce Ellis, Arlington, VA, USA; Natalie Madero, Baltimore, MD, USA; Emily Reid, Durham, NC, USA; Donna Little, Baltimore; April Greek, Seattle, WA, USA; Dale Rhoda, Linda Delma Gieseke, Stephanie Ashcraft, Columbus, OH, USA; Battelle Toxicology Northwest, Richland, WA (Katherine Gideon); Carolinas Rehabilitation, Charlotte, NC (Tara Ruhlen); Centers for Disease Control and Prevention, Atlanta, GA, USA (Mariela Z Scarbrough, Trevor Thompson, Deblina Datta, Susan Hariri); Louisiana State University, Baton Rouge, LA, USA (Lauren Cole); Michigan Department of Community Health, Lansing, MI, USA (Lana Ashley, Jetty Alverson, Michelle Hulbert, Won Silva); National Cancer Institute, Rockville, MD (Sean Altekruse); St Joseph Mercy Hospital, Ann Arbor, MI (Samuel Hirsch); University of Florida, Gainesville, FL, USA (Martha Campbell-Thompson, Amy Wright, Kelley Durden); University of Miami, Florida Cancer Data System, Miami, FL (Jill MacKinnon, Carlos Alvarez); University of Hawaii, Cancer Research Center of Hawaii, Honolulu, HI, USA (Hugh Luk, David Horio, Shoji Ikeda, Michael Green, Catherine Grafel-Anderson, Rayna Weise); University of Iowa, Iowa City, IA, USA (Freda Selk, Dan Olson); University of Kentucky, Lexington, KY, USA (Thomas Tucker, Amy Christian); University of North Carolina Hospitals Cancer Registry, Chapel Hill, NC (Susan Brossoie); and University of Southern California, Los Angeles, CA, USA: Joe House, Myles G. Cockburn, Andre Kim)

Acknowledgment

We thank all members of the HPV Typing of Cancers Workgroup for their contributions toward this study.

The collection of original specimens from nonrepositories (Kentucky, Florida, Michigan, Louisiana), coordination of genotyping data from both the Surveillance, Epidemiology, and End Results (SEER) program and the National Program of Cancer Registries (NPCR) was largely supported by CDC intramural funds and Vaccine For Children Funds. This project has been supported in part with federal funds by CDC under grant nos. 5U58DP000810-5 (Kentucky), 5U58DP000844-5 (Florida), 5U58DP000812-5 (Michigan), and 5U58DP000769-5 (Louisiana); and with federal funds for Residual Tissue Repositories from the National Cancer Institute SEER Population-based Registry Program, National Institutes of Health, Department of Health and Human Services, under contract nos. N01-PC-35139 (Los Angeles), N01-PC-35143 (Iowa), and N01-PC-35137 (Hawaii).

The collection of data from California used in this publication was largely supported by the California Department of Health Services as part of the statewide cancer reporting program mandated by California Health and Safety Code Section 103885; by the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, under contract no. N01-PC-2010-00035; and grant no. 1U58DP000807-3 from CDC.

B.Y.H. has received consultation and speaker fees from Merck and Co., Inc.

Dr Steinau is a microbiologist at the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA. He leads the human papillomavirus DNA laboratory in the Chronic Viral Diseases Branch. His main interests focus on molecular methods for detection and characterization of human papillomaviruses and associated diseases.

References

1. de Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. Lancet Oncol. 2012;13:607–15. http://dx.doi.org/10.1016/S1470-2045(12)70137-7
2. Centers for Disease Control and Prevention (CDC). Human papillomavirus-associated cancers—United States, 2004–2008. MMWR Morb Mortal Wkly Rep. 2012;61:258–61.
3. Gillison ML. Human papillomavirus–associated head and neck cancer is a distinct epidemiologic clinical, and molecular entity. Semin Oncol. 2004;31:744–54. http://dx.doi.org/10.1053/j.seminoncol.2004.09.011
4. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med. 2007;356:1944–56. http://dx.doi.org/10.1056/NEJMoa065497
5. Martinez I, Wang J, Hopson KF, Ferris RL, Khan SA. Identification of differentially expressed genes in HPV-positive and HPV-negative oropharyngeal squamous cell carcinomas. Eur J Cancer. 2007;43:415–32. http://dx.doi.org/10.1016/j.ejca.2006.09.001
6. Miller DL, Puricelli MD, Stack MS. Virology and molecular pathogenesis of HPV (human papillomovirus)-associated oropharyngeal squamous cell carcinoma. Biochem J. 2012;443:339–53. http://dx.doi.org/10.1042/BJ20112017
7. Herrero R, Quint W, Hildesheim A, Gonzalez P, Struijk L, Katki HA, et al. Reduced prevalence of oral human papillomavirus (HPV) 4 years after HPV vaccination in a randomized clinical trial in Costa Rica. PLoS ONE. 2013;8:e68329. http://dx.doi.org/10.1371/journal.pone.0068329

8. Watson M, Saraiya M, Ahmed F, Cardinez CJ, Reichman ME, Weir HK, et al. Using population-based cancer registry data to assess the burden of human papillomavirus-associated cancers in the United States: overview of methods. Cancer. 2008;113 (Suppl):2841–54. http://dx.doi.org/10.1002/cncr.23758
9. Gillison ML, Chaturvedi AK, Lowy DR. HPV prophylactic vaccines and the potential prevention of noncervical cancers in both men and women. Cancer. 2008;113:3036–46. http://dx.doi.org/10.1002/cncr.23764
10. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst. 2008;100:261–9. http://dx.doi.org/10.1093/jnci/djn011
11. Kreimer AR, Clifford GM, Boyle PB, Fransceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev. 2005;14:467–75. http://dx.doi.org/10.1158/1055-9965.EPI-04-0551
12. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol. 2011;29:4294–301. http://dx.doi.org/10.1200/JCO.2011.36.4596
13. Ryerson AB, Peters ES, Caughlin SS, Chen VW, Gillison ML, Reichman ME, et al. Burden of potentially human papillomavirus-associated cancer of the oropharynx and oral cavity in the US, 1998–2003. Cancer. 2008;113:2901–9. http://dx.doi.org/10.1002/cncr.23745
14. Mirghani H, Moreau F, Lefèvre M, Tam C, Périé S, Soussan P, et al. Human papillomavirus type 16 oropharyngeal cancer in lymph nodes as a marker of metastases. Arch Otolaryngol Head Neck Surg. 2011;137:910–4. http://dx.doi.org/10.1001/archoto.2011.141
15. Gargano JW, Wilkinson EJ, Unger ER, Steinau M, Watson M, Huang Y, et al. Prevalence of human papillomavirus types in invasive vulvar cancers and vulvar intraepithelial neoplasia 3 in the United States before vaccine introduction. J Low Genit Tract Dis. 2012;16:471–9. http://dx.doi.org/10.1097/LGT.0b013e3182472947
16. Steinau M, Unger ER, Hernandez BY, Goodman MT, Copeland G, Hopenhayn C, et al. Human papillomavirus prevalence in invasive anal cancers in the United States prior to vaccine introduction. J Low Genit Tract Dis. 2013; 17:397-403. http://dx.doi.org/10.1097/LGT.0b013e31827ed372
17. Steinau M, Patel SS, Unger ER. Efficient DNA extraction for HPV genotyping in formalin-fixed, paraffin-embedded tissues. J Mol Diagn. 2011;13:377–81. http://dx.doi.org/10.1016/j.jmoldx.2011.03.007
18. Hariri S, Steinau M, Rinas A, Gargano J, Ludema C, Unger ER, et al. HPV genotyping in high grade cervical lesions and invasive cervical carcinoma as detected by two commercial DNA assays, North Carolina, 2001–2006. PLoS ONE. 2012;7:e34044. http://dx.doi.org/10.1371/journal.pone.0034044
19. Steinau M, Onyekwuluje JM, Scarbrough MZ, Unger ER, Dillner J, Zhou T. Performance of commercial reverse line blot assays for human papillomavirus genotyping. J Clin Microbiol. 2012;50:1539–44. http://dx.doi.org/10.1128/JCM.06576-11
20. Onyekwuluje JM, Steinau M, Swan DC, Unger ER. A real time PCR assay for HPV52 detection and viral load quantification. Clin Lab. 2012;58:61–6.
21. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah K, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999;189:12–9. http://dx.doi.org/10.1002/(SICI)1096-9896(199909)189:1<12:AID-PATH431>3.0.CO;2-F
22. Watson M, Lyu C, Unger ER, Copeland G, Peters E, Huang Y, et al. Centers for Disease Control and Prevention human papillomavirus typing of cancers study with 7 registries: evaluating representativeness. North American Association of Central Cancer Registries (NAACCR) Annual Conference, 2011. Abstract, no. 58. http://www.naaccr.org/LinkClick.aspx?fileticket=udtv3Esreek%3d&tabid=257&mid=732
23. Mehanna H, Beech T, Nicholson T, El-Hariry I, McConkey C, Paleri V, et al. Prevalence of human papillomavirus in oropharyngeal and nonoropharyngeal head and neck cancer-systematic review and meta-analysis of trends by time and region. Head Neck. 2013;35:747–55. http://dx.doi.org/10.1002/hed.22015
24. Frisch M, Hjalgrim H, Jaeger AB, Biggar RJ. Changing patterns of tonisllar squamous cell carcinoma in the United States. Cancer Causes Control. 2000;11:489–95. http://dx.doi.org/10.1023/A:1008918223334
25. Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol. 2009;45:309–16. http://dx.doi.org/10.1016/j.oraloncology.2008.06.002
26. Paolini F, Rizzo C, Sperduti I, Pichi B, Mafera B, Rahimi SS, et al. Both mucosal and cutaneous papillomaviruses are in the oral cavity but only alpha genus seems to be associated with cancer. J Clin Virol 2013;56:72–6 http://dx.doi.org/10.1016/j.jcv.2012.09.016
27. Gillison ML, Broutian T, Pickard RK, et al. Prevalence of oral HPV infection in the United States, 2009–2010. JAMA. 2012;307:693–703. http://dx.doi.org/10.1001/jama.2012.101
28. Brown LM, Check DP, Devesa SS. Oropharyngeal cancer incidence trends: diminishing disparities. Cancer Causes Control. 2011;22:753–63. http://dx.doi.org/10.1007/s10552-011-9748-1
29. Settle K, Posner MR, Schumaker LM, Tan M, Suntharalingam M, Goloubeva O, et al. Racial survival disparity in head and neck cancer results from low prevalence of human papillomavirus infection in black oropharyngeal cancer patients. Cancer Prev Res (Phila). 2009;2:776–81. http://dx.doi.org/10.1158/1940-6207.CAPR-09-0149
30. Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. J Clin Oncol. 2008;26:612–9. http://dx.doi.org/10.1200/JCO.2007.14.1713
31. Pai SI, Westra WH. Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. Annu Rev Pathol. 2009;4:49–70. http://dx.doi.org/10.1146/annurev.pathol.4.110807.092158
32. Jung AC, Briolat J, Millon R, de Reyniès A, Rickman D, Thomas E, et al. Biological and clinical relevance of transcriptionally active human papillomavirus (HPV) infection in oropharynx squamous cell carcinoma. Int J Cancer. 2010;126:1882–94. http://dx.doi.org/10.1002/ijc.24911
33. Sethi S, Ali-Fehmi R, Franceschi S, Struijk L, van Doorn L-J, Quint W, et al. Characteristics and survival of head and neck cancer by HPV status: a cancer registry-based study. Int J Cancer. 2012;131:1179–86. http://dx.doi.org/10.1002/ijc.26500

Address for correspondence: Martin Steinau, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, mailstop G41, Atlanta, GA, USA 30333-4027; email: MSteinau@cdc.gov

Factors Associated with Antimicrobial Drug Use in Medicaid Programs

Pengxiang Li, Joshua P. Metlay, Steven C. Marcus, and Jalpa A. Doshi

Using US Medicaid data, we found that 52% of adult Medicaid patients with acute respiratory tract infections filled prescriptions for antimicrobial drugs in 2007. Factors associated with lower likelihood of use were higher county-level availability of primary care physicians and state-level participation in a campaign for appropriate antimicrobial drug use.

Antimicrobial drugs are not recommended for the treatment of acute respiratory tract infections (ARIs), such as colds, upper respiratory tract infections (URIs), and acute bronchitis (*1*,*2*). Unnecessary use contributes to emergence of antimicrobial drug–resistant bacteria (*1*), an emerging public health crisis (*2*) that contributes to greater rates of illness and death and economic costs as high as $4 billion/year (*3*).

Inappropriate use of antimicrobial drugs in Medicaid programs is a potentially serious problem (*4*,*5*). Medicaid is a US health insurance program that covers 58 million low-income persons and families (*6*). The number of enrolled adults is expected to increase substantially as a result of the Patient Protection and Affordable Care Act (*7*). In this study, we estimated the rate and factors associated with antimicrobial drug use for the treatment of ARIs among adult Medicaid enrollees.

The Study

We used the 2007 Medicaid Analytic Extract files for patients ≥21 years of age from 40 states linked with the Area Resource File. Index visits were identified as the first visit to a physician during the study period when a primary diagnoses of ARI was made (cold, acute URIs at multiple unspecified sites, or acute bronchitis) (*8*,*9*). The identification period for the index visit was January 1, 2007, through December 24, 2007. We excluded patients who received index visit diagnoses for which antimicrobial drugs were appropriate (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/5/13-0493-Techapp1.pdf).

Author affiliation: University of Pennsylvania, Philadelphia, Pennsylvania, USA

DOI: http://dx.doi.org/10.3201/eid2005.130493

The outcome variable was presence or absence of a claim for an antimicrobial drug prescription linked to the index visit. Drug classes considered were cephalosporins, penicillins, sulfonamides, macrolides (including azalides), lincosamides, tetracyclines, and quinolones (*4*). Similar to most prescription claims data, Medicaid drug claims do not list a diagnosis that corresponds to the indication for treatment. Hence, the drug was presumed to have been prescribed for an ARI if the prescription was filled on the same date that the patient visited the physician for the ARI or within 4 days of this index visit (*8*).

Logistic regression analyses with robust estimation, adjusting for state-level clustering, were used to identify factors associated with antimicrobial drug prescriptions for ARI visits. Covariates included patient age, sex, race, and the Prescription Drug Hierarchical Coexisting Condition score as a measure of concurrent conditions and medication need (*10*) and county-level covariates. To account for seasonal effects, we included indicators for the quarter in which the index visit occurred. The density of primary care physicians in the county of the beneficiary's residence was measured from the Area Resource File as the number of general practice, family medicine, and general internal medicine physicians per 10,000 persons. This measure was coded as categorical variables according to the quintile of the measure across all counties in the Area Resource File. An indicator variable identified whether the patient resided in a state that was funded by the Centers for Disease Control and Prevention (CDC) Get Smart: Know When Antibiotics Work campaign for appropriate antimicrobial drug use within the 5 years before the study (*11*). Sensitivity analyses varied the covariates included in the regression analyses and the time window (3–7 days after index visit) for linking the drugs to the URI diagnosis (*5*,*9*). Subgroup analyses were conducted among patients ≤65years of age and patients without diabetes or congestive heart failure.

In 2007, a total of 194,874 adults had at least 1 physician visit at which a primary diagnosis of ARI was made with no other associated secondary diagnoses for which treatment with an antimicrobial drug would be appropriate (Table 1). After this visit, ≈52% of patients filled an antimicrobial drug prescription (Figure). The most common prescriptions filled were for macrolides (27.8%) and penicillins (12.3%).

Odds of filling antimicrobial drug prescriptions for treatment of ARI were significantly lower for older adults, men, and nonwhite patients (Table 2). Patients with acute bronchitis were substantially more likely than patients with a cold or URI to fill these prescriptions (69% vs. 40%; odds ratio [OR] 3.32; 95% CI 2.78–3.95). Odds of filling antimicrobial drug prescriptions were significantly lower for patients residing in a county for which the quintile for primary care physician density was highest than for patients in a county for which the quintile was lowest (48.2%

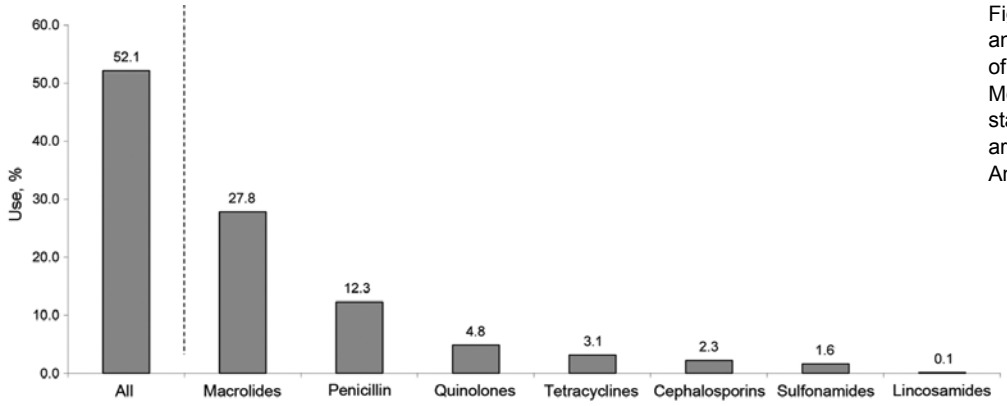


Figure. Percentage of antimicrobial drug use, by type of agent, among 194,874 adult Medicaid patients in 40 US state Medicaid programs. Data are from the 2007 Medicaid Analytic Extract files.

Table 1. Characteristics of 194,874 adult Medicaid patients with acute respiratory tract infection, 40 US states, January 1,–December 24, 2007*

Variable	No. (%) or mean (SD)
Age, y, no. (%)	
21–29	45,447 (23.3)
30–39	42,977 (22.1)
40–49	42,924 (22)
50–59	38,376 (19.7)
60–64	16,929 (8.7)
≥65	8,221 (4.2)
Sex, no. (%)	
F	143,329 (73.5)
M	51,545 (26.5)
Race, no. (%)	
White	100,310 (51.5)
Black	46,282 (23.7)
Hispanic	16,404 (8.4)
Other	31,878 (16.4)
Diagnosis at index visit, no. (%)	
Cold or acute URIs (ICD-9 codes 460 and 465)	113,394 (58.2)
Acute bronchitis (ICD-9 code 466)	81,480 (41.8)
RxHCC score, mean (SD)†	0.5 (0.6)
Quarter of index visit date, no. (%)	
Jan–Mar	85,601 (43.9)
Apr–Jun	36,771 (18.9)
Jul–Sep	30,807 (15.8)
Oct–Dec	41,695 (21.4)
Residence in low-education county, no. (%)‡	
No	144,335 (74.1)
Yes	50,539 (25.9)
County-level annual per capita income, mean (SD)	32,700 (15.6)
Residence in urban area, no. (%)	
No	65,766 (33.7)
Yes	129,108 (66.3)
Residence in state participating in CDC Get Smart campaign, no. (%)§	
No	38,332 (19.7)
Yes	156,542 (80.3)
Primary care physicians/10,000 persons in county, no. (%)¶	
<2.2	14,816 (7.6)
2.2–3.4	28,500 (14.6)
3.5–4.7	34,017 (17.5)
4.8–6.5	47,460 (24.4)
>6.5	70,081 (36)

*Data from the 2007 Medicaid Analytic Extract files linked with the Area Resource File. URI, upper respiratory tract infection; ICD-9, International Classification of Diseases, Ninth Revision; RxHCC, prescription drug Hierarchical Coexisting Condition; CDC, Centers for Disease Control and Prevention.
†Modified RxHCC score used here, wherein coefficients for age and sex are zeroed out in the score calculation because regression models separately control for these variables. Range in sample described here 0–5.3. A higher score indicates a higher medical comorbidity burden.
‡County with ≥25% adults without a high school diploma.
§In this sample, 33 of 40 states participated in the CDC Get Smart campaign during 2002–2006.
¶Categories were based on quintile of county-level number of primary care physicians/10,000 persons. Each category includes 644 counties.

Table 2. Factors associated with antimicrobial drug use among 194,874 adult Medicaid patients, 40 US states, 2007*

Variable	% Visits at which drugs were prescribed	Odds ratio (95% CI)
Age, y		
21–29	49.8	Referent
30–39	53.7	1.10 (1.05–1.16)
40–49	53.8	1.09 (0.99–1.20)
50–59	53.3	1.09 (0.96–1.23)
60–64	51.3	1.02 (0.89–1.16)
≥65	43.2	0.77 (0.63–0.94)
Sex		
F	52.4	Referent
M	51.1	0.94 (0.90–0.99)
Race		
White	56.9	Referent
Black	48.9	0.82 (0.74–0.91)
Hispanic	45.6	0.73 (0.64–0.82)
Other	45.0	0.75 (0.61–0.92)
Diagnosis at index visit		
Cold or acute URI, ICD-9 codes 460 and 465	40.0	Referent
Acute bronchitis, ICD-9 code 466	69.0	3.32 (2.78–3.95)
RxHCC score†		0.94 (0.88–1.00)
Quarter of index visit date		
Jul–Sep	52.8	Referent
Jan–Mar	52.3	1.04 (1.01–1.08)
Apr–Jun	52.1	1 (0.97–1.04)
Oct–Dec	51.1	0.98 (0.95–1.01)
Residence in low-education county‡		
No	52.1	Referent
Yes	52.1	0.96 (0.85–1.09)
County–level annual per capita income (in $1,000)§		1.00 (1.00–1.00)
Residence in urban area		
No	55.8	Referent
Yes	50.2	0.91 (0.82–1.00)
Residence in state participating in CDC Get Smart campaign¶		
No	57.7	Referent
Yes	50.7	0.74 (0.62–0.88)
No. primary care physicians/10,000 persons in county#		
<2.2	56.8	Referent
2.2–3.4	56.2	0.96 (0.87–1.07)
3.5–4.7	55.4	0.91 (0.80–1.04)
4.8–6.5	51.4	0.84 (0.73–0.96)
>6.5	48.2	0.76 (0.66–0.88)

*Data are from the 2007 Medicaid Analytic Extract files linked with the Area Resource File. URI, upper respiratory tract infection; ICD-9, International Classification of Diseases, Ninth Revision; RxHCC, prescription drug Hierarchical Coexisting Condition; CDC, Centers for Disease Control and Prevention.
†Modified RxHCC score used here, wherein coefficients for age and sex are zeroed out in the score calculation because regression models separately control for these variables. Range in sample described here 0–5.3. A higher score indicates a higher medical comorbidity burden. Odds ratio indicates the increased odds of antimicrobial drug use associated with per unit increase in the score.
‡The categories were based on quintile of county-level number of PCP physicians per 10,000 persons. Each category includes 644 counties.
§Defined as a county with ≥25% adults without a high school diploma.
¶In separate analysis, the county-level annual per capita income was coded as categorical variables according to the quintile of the measure across all counties in the Area Resource File. Similar to the continuous variables, the categorical variables were not significant.
#In our sample, 33 of 40 states participated in the CDC Get Smart campaign during 2002–2006.

vs. 56.8%; OR 0.76, 95% CI 0.66–0.88). Likelihood of filling antimicrobial drug prescriptions was lower for patients in 33 states that had participated in the CDC Get Smart campaign during 2002–2006 than for those in other states (50.7% vs. 57.7%; OR 0.74, 95% CI 0.62–0.88). Results of all sensitivity and subgroup analyses were consistent with main results (online Technical Appendix Table 1).

Conclusion

In 2007, more than half of Medicaid patients filled a prescription for antimicrobial drugs for an ARI, despite essentially no evidence of efficacy for this use. The substantially higher use of antimicrobial drugs for acute bronchitis than for colds or other URIs raises the need for effective interventions to further support physician decision making. Examples of such interventions include active clinician education strategies (e.g., academic detailing, educational workshops, and consensus-building sessions), which are more effective than passive education strategies (e.g., distribution of educational materials) (*1*).

Lower availability of primary care physicians might be associated with higher rates of antimicrobial drug

prescribing for ARIs, given that clinicians in areas with fewer primary care physicians see more patients. Physicians with greater patient workloads might be more likely to prescribe antimicrobial drugs for ARIs (*1*), given that they do not have time to counsel patients against use of antimicrobial drugs. This finding has implications for Medicaid because enrollment is expected to increase substantially by 2019 under the Patient Protection and Affordable Care Act. Whether the number of primary care physicians will be adequate to meet this increased demand is a serious concern. In addition, a recent survey showed that nearly one third of physicians are unwilling to see new Medicaid patients (*12*). As a result, inappropriate antimicrobial drug use in Medicaid programs might increase, especially where primary care physician density is low.

Use of antimicrobial drugs for ARIs was lower among patients in states that participated in the CDC Get Smart campaign during 2002–2006. Under this program, CDC helped fund development and implementation of local campaigns to promote appropriate use of antimicrobial drugs. Audiences included providers and patients (*11*). Adding patient education to an existing physician-centered intervention reduces antimicrobial drug use among adults with acute bronchitis (*13*). This finding suggests that such public health campaigns might be associated with lower unnecessary antimicrobial drug use.

Among study limitations are use of administrative claims data, which are collected for purposes of payment rather than research; thus, coding of URI diagnoses might be questionable. Nevertheless, numerous claims-based studies have identified URIs by using International Classification of Diseases, Ninth Revision codes in claims data (*8*,*9*), and a validation study that used chart review showed that for URIs, specificity for these codes was >0.97 (95% CI 0.95–0.98) and sensitivity was 0.56 (95% CI 0.45–0.67) (*14*). Given the cross-sectional study design, our findings associated with primary care physician density and the CDC Get Smart campaign cannot be considered causal. Furthermore, our use of prescription-fill data as a proxy for medication use might have overestimated usage rates. However, this approach has been validated and widely used in studies of medication use (*15*).

That a high percentage of adult Medicaid enrollees with ARIs received antimicrobial drugs unnecessarily raises concern about further widespread use with the upcoming expansion in Medicaid enrollment under health care reform. Clinicians, public health officials, and policymakers should consider ways to curb inappropriate antimicrobial drug use in this population.

Dr Li is a senior research investigator at the Division of General Internal Medicine and a senior fellow at the Leonard Davis Institute of Health Economics, both at the University of Pennsylvania. His research interests include pharmaceuticals and health services.

References

1. Ranji S, Steinman M, Shojania K, Sundaram V, Lewis R, Arnold S, et al. Closing the quality gap: a critical analysis of quality improvement strategies. Technical review 9–antibiotic prescribing behavior, vol. 4, publication no. 04 (06)-0051–4. Rockville (MD): Agency for Healthcare Research and Quality; 2006.
2. Gonzales R, Steiner JF, Sande MA. Antibiotic prescribing for adults with colds, upper respiratory tract infections, and bronchitis by ambulatory care physicians. JAMA. 1997;278:901. http://dx.doi.org/10.1001/jama.1997.03550110039033
3. Grijalva CG, Nuorti JP, Griffin MR. Antibiotic prescription rates for acute respiratory tract infections in US ambulatory settings. JAMA. 2009;302:758–66. http://dx.doi.org/10.1001/jama.2009.1163
4. Brown DW, Taylor R, Rogers A, Weiser R, Kelley M. Antibiotic prescriptions associated with outpatient visits for acute upper respiratory tract infections among adult Medicaid recipients in North Carolina. N C Med J. 2003;64:148–56.
5. Zuckerman IH, Perencevich EN, Harris AD. Concurrent acute illness and comorbid conditions poorly predict antibiotic use in upper respiratory tract infections: a cross-sectional analysis. BMC Infect Dis. 2007;7:47. http://dx.doi.org/10.1186/1471-2334-7-47
6. Kaiser Family Foundation. The Medicaid program at a glance [cited 2011 Sep 15]. http://www.kff.org/medicaid/upload/7235-04.pdf
7. Holahan J, Headen I. Medicaid coverage and spending in health reform: national and state-by-state results for adults at or below 133% FPL. Washington (DC): The Kaiser Commission on Medicaid and the Uninsured; 2011.
8. Mainous AG III, Zoorob RJ, Hueston WJ. Current management of acute bronchitis in ambulatory care: the use of antibiotics and bronchodilators. Arch Fam Med. 1996;5:79–83. http://dx.doi.org/10.1001/archfami.5.2.79
9. Li J, De A, Ketchum K, Fagnan LJ, Haxby DG, Thomas A. Antimicrobial prescribing for upper respiratory infections and its effect on return visits. Fam Med. 2009;41:182–7.
10. Robst J, Levy JM, Ngber MJ. Diagnosis-based risk adjustment for Medicare prescription drug plan payments. Health Care Financ Rev. 2007;28:15–30.
11. Centers for Disease Control and Prevention. Get Smart: know when antibiotics work [cited 2012 Feb 1]. http://www.cdc.gov/getsmart/index.html
12. Decker SL. In 2011 nearly one-third of physicians said they would not accept new Medicaid patients, but rising fees may help. Health Aff (Millwood). 2012;31:1673–9. http://dx.doi.org/10.1377/hlthaff.2012.0294
13. Gonzales R, Corbett KK, Leeman-Castillo BA, Glazner J, Erbacher K, Darr CA, et al. The "minimizing antibiotic resistance in Colorado" project: impact of patient education in improving antibiotic use in private office practices. Health Serv Res. 2005;40:101–16. http://dx.doi.org/10.1111/j.1475-6773.2005.00344.x
14. Beitel AJ, Olson KL, Reis BY, Mandl KD. Use of emergency department chief complaint and diagnostic codes for identifying respiratory illness in a pediatric population. Pediatr Emerg Care. 2004;20:355–60. http://dx.doi.org/10.1097/01.pec.0000133608.96957.b9
15. Steiner JF, Prochazka AV. The assessment of refill compliance using pharmacy records: methods, validity, and applications. J Clin Epidemiol. 1997;50:105–16. http://dx.doi.org/10.1016/S0895-4356(96)00268-5

Address for correspondence: Pengxiang Li, Division of General Internal Medicine, University of Pennsylvania, 423 Guardian Dr, Blockley Hall Room 1215, Philadelphia, PA 19104, USA; email: penli@mail.med.upenn.edu

Chronic Wasting Disease Agents in Nonhuman Primates

Brent Race, Kimberly D. Meade-White, Katie Phillips, James Striebel, Richard Race, and Bruce Chesebro

Chronic wasting disease, a prion disease of cervids, may infect humans, but this is unproven. Primates from 2 genera were observed for 9–10 years after intracerebral or oral inoculation. Cynomologus macaques were completely resistant. However, squirrel monkeys were highly susceptible to the pathogen, which adapted more quickly on second passage.

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy that can infect mammals in the family Cervidae, which includes deer, elk, and moose, among other species. Initially detected in a captive deer in 1967, the disease is now widespread in the United States, Canada, and South Korea (*1*). Because cervids are commonly consumed as food by humans and other mammals, the cross-species potential of the causal pathogen must be determined. CWD prions injected intracerebrally have infected agricultural animals and scavengers (*2*); however, transgenic mice that expressed human prion protein (PrP) were not susceptible (*3*–*6*) through this route. We previously analyzed the susceptibility of 2 genera of nonhuman primates to CWD agents by intracerebral and oral routes (*7*). The results showed a high attack rate in squirrel monkeys (*Saimiri sciureus*) inoculated intracerebrally but a low attack rate and long incubation periods by those exposed orally. In contrast, no cynomolgus macaques (*Macaca fascicularis*) showed clinical signs of transmissible spongiform encephalopathy (TSE) when exposed by either route. The long incubation periods observed in squirrel monkeys prompted us to observe the remaining monkeys for >4 additional years. Here we provide an update and report results of new experiments showing that squirrel monkey–adapted CWD (SM-CWD) has an accelerated incubation period on second passage.

The Study

Squirrel monkeys were inoculated intracerebrally or orally with CWD inocula (*7*). We initially reported that 11/13 intracerebrally infected monkeys were euthanized at 41 months postinoculation (mpi) on average, and disease developed in 2/12 orally infected squirrel monkeys on average of 69 mpi (*7*). Disease developed in the 2 remaining intracerebrally infected squirrel monkeys at 61 and 75 mpi, respectively, changing the intracerebral attack rate to 100% (Figure 1, Table 1). Of the 10 remaining orally inoculated squirrel monkeys, disease developed in 9, bringing the overall oral attack rate to 92% and the average incubation period to 68 mpi (Figure 1, Table 1). Clinical signs were subtle; the most prominent finding was gradual weight loss (Table 1). A final diagnosis of CWD agent infection was made by using immunoblotting and immunohistochemical testing to determine accumulation of abnormal, disease-associated prion protein (PrPres) in brain tissue (wwwnc.cdc.gov/EID/article/20/5/13-0778-Techapp1.pdf).

To compare the neuropathologic changes in intracerebrally and orally infected squirrel monkeys, we analyzed 10 brain regions for spongiform lesion severity and PrPres deposition (Figure 1, panels B, C). No statistically significant differences were noted between the 2 routes of infection ($p<0.05$). All squirrel monkeys studied had severe spongiform degeneration in the striatum (Figure 1, panel D) and little involvement in cerebellum and occipital lobes (Figure 1, panel E). Spongiform lesions in cortical gray matter were not consistent throughout the brain. Affected areas were commonly observed adjacent to normal regions, most frequently in the frontal, temporal, and parietal lobes (Figure 1, panel F). Except for the striatum, PrPres deposition was generally most prominent in areas that showed severe vacuolation. PrPres deposits appeared in 2 forms: dense punctate extracellular plaques (Figure 1, panel G) and less dense pericellular aggregates. The spleens of CWD agent–infected squirrel monkeys were positive for PrPres in 46% of intracerebrally infected and 60% of orally infected squirrel monkeys (Figure 1, panel H). At least 1 lymph node was positive in 30% of intracerebrally infected squirrel monkeys and in 40% of orally infected squirrel monkeys (Figure 1, panel J; Technical Appendix).

Of the squirrel monkeys under study, 3 *PRNP* genotypes were represented (*7*). In the group of orally infected squirrel monkeys, 3 had a unique heterozygous genotype that encoded either 4 or 5 octapeptide repeats. Two of these monkeys were the last orally infected monkeys to be euthanized because of clinical disease (80 and 107 mpi), and the third heterozygote was clinically normal at 108 mpi. Heterozygosity within the *PRNP* gene has been shown to delay or prevent prion disease (*8*) and may play a role in this study.

We inoculated cynomolgus macaques as another nonhuman primate model for cross-species transmission of CWD. Compared with squirrel monkeys, cynomolgus

Author affiliation: National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA

DOI: http://dx.doi.org/10.3201/eid2005.130778

macaques are biologically closer to humans, and cynomolgus macaque PrP is more homologous to human PrP (*7*). Nine cynomolgus macaques were inoculated orally and 6 were inoculated intracerebrally with 1 of 3 CWD pools as described (*7*). Our first report included negative data from 1 cynomolgus macaque euthanized at 49 mpi (*7*). Since then, we have euthanized and screened 6 cynomolgus macaques for TSE (Table 2). No evidence of prion infection was detected by immunoblot and immunohistochemical methods (data not shown).

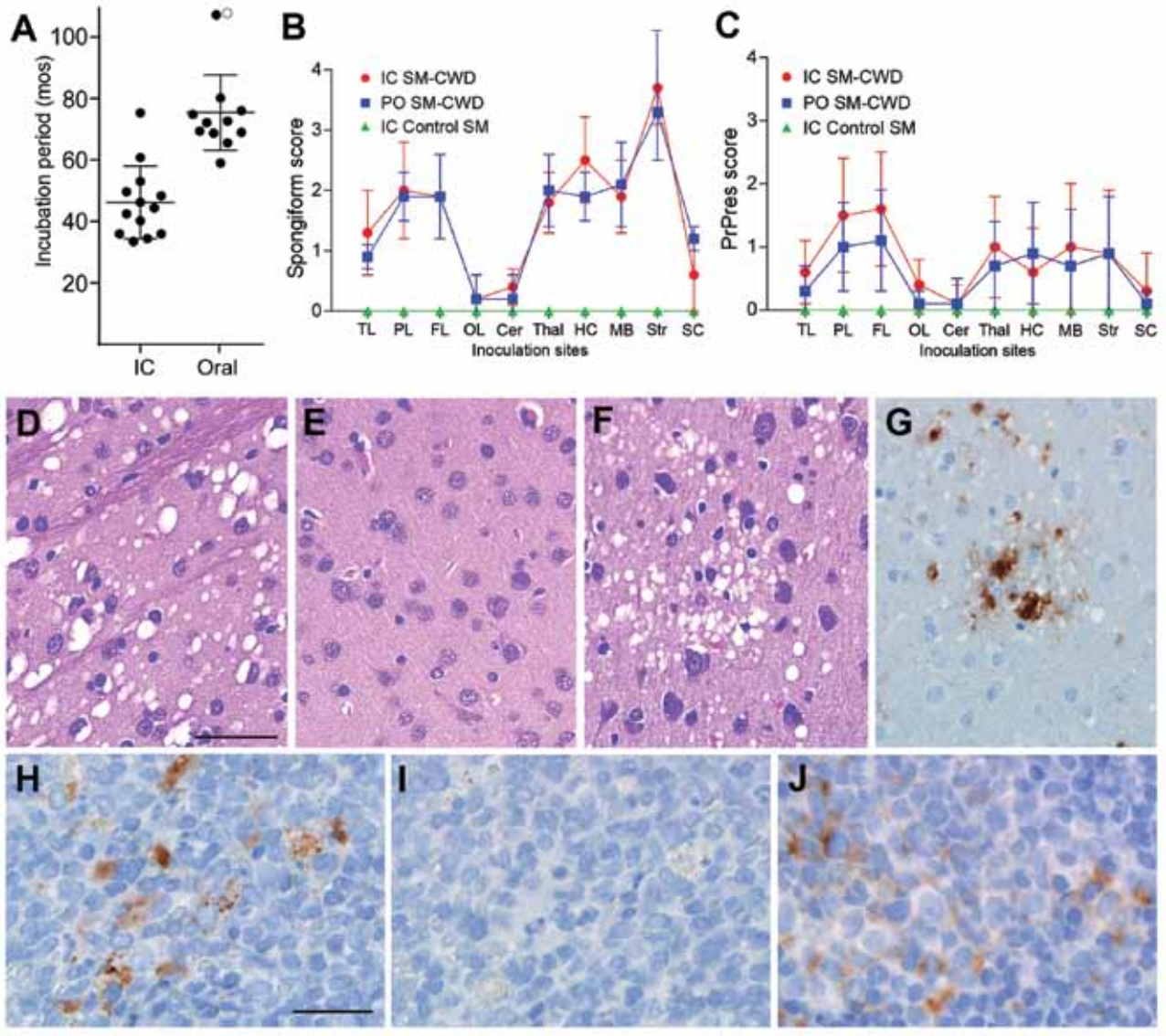


Figure 1. Incubation periods of chronic wasting disease (CWD) and neuropathologic features of CWD agent–infected squirrel monkeys. A) Incubation periods for squirrel monkeys infected with CWD agents by intracerebral (IC) or oral (PO) routes. Solid circles indicate euthanized squirrel monkeys (SM) that tested positive for prion disease. The open circle indicates 1 squirrel monkey that remained clinically normal at 108 months postinoculation (mpi). Lines indicate the mean and standard deviation within each group. B, C) Lesion profiles of CWD-agent–infected squirrel monkeys showing spongiform degeneration (B) and PrPres deposition (C) values in 10 gray matter regions of the brain. N values for each group are as follows: IC SM-CWD, 11; PO SM-CWD, 7; IC control SM, 1. TL, temporal lobe; PL, parietal lobe; FL, frontal lobe; OL, occipital lobe; Cer, cerebellum; Thal, thalamus; HC, hippocampus; MB, midbrain; Str, stiatum; SC, spinal cord. Error bars show the SD for each group. Panels D–G show brain from a squirrel monkey infected PO with CWD and euthanized at 69 months postinoculation. Panels D–F are stained with hematoxylin and eosin and show D) severe spongiform lesions in the striatum, E) lack of pathology in the occipital lobe, and F) pathology in the parietal lobe. Panels G, H, and J show immunohistochemical staining for PrPres by using anti-PrP antibody D13. G) Adjacent section to the region depicted in F shows the positive correlation of PrPres (brown) with spongiform degeneration. Panels H–J show lymphoid tissue from a squirrel monkey infected PO with CWD and euthanized at 80 mpi. H) PrPres (brown) staining in spleen and J) mesenteric lymph node. I) No primary antibody control of the region shown in H, demonstrating specificity of stain observed in H. The scale bar shown in D applies to panels D–G and represents 50 µm; the scale bar shown in H applies to H–J and represents 25 µm.

Table 1. Squirrel monkeys inoculated with CWD or squirrel monkey–adapted CWD agents*

Disease incidence†	Inoculum‡	Route of inoculation	Titer inoculated§	Incubation days, range, (avg)¶	Weight change range, % (avg,%)
13/13	MD-1,2,3 Elk-1,2,3 WTD-1,2	Intracerebral	1.3×10^5-1.0×10^7	33–75 (46)	−8 to −43 (−29.5)
11/12#	MD-1,3 Elk-1,2,3 WTD-1,2	Oral	9.6×10^7-1.5×10^9	59–107 (68)	−8 to −41 (−28)
2/2	SM-CWD	Intracerebral	NA	23–24 (23.5)	−8 to −21 (−14.5)
0/1	Normal elk	Intracerebral	NA	82 NS	0
0/1	Buffer control	Oral	NA	>108	−6
0/1	Normal elk	Oral	NA	123 NS	+7

*An early version of some of these data is shown in Tables 1, 2 of (*7*). Since that time more infected animals have been euthanized and the data have been updated. CWD, chronic wasting disease; MD, mule deer; WTD, white-tailed deer; NA, not applicable; NS, no clinical transmissible spongiform encephalopathy signs.
†Number of monkeys in which prion disease developed/number inoculated.
‡Several different inocula were used for this study. Each individual animal was inoculated with 1 inoculum. Detailed descriptions can be found in (*7*).
§Infectivity titers were determined by using endpoint dilution titer in transgenic deer PrP mice and are listed as 50% infectious dose/gram of brain.
¶The range of incubation periods observed is shown as months postinoculation followed with the average incubation period of the group in parentheses. Monkeys listed as NS did not show any clinical signs compatible with transmissible spongiform encephalopathy.
#Three monkeys from this group are not included in this calculation because they were euthanized before 45 months postinoculation for reasons unrelated to transmissible spongiform encephalopathy disease. The sole remaining animal in this group appeared normal at 108 months postinoculation.

The lack of CWD transmission during >10 years suggests that a substantial species barrier exists between cervids and cynomolgus macaques. In most TSE animal models, PrPres can be detected by 1/3–1/2 of the known incubation periods. If we extrapolate this to the cynomolgus macaques in this study, negative test results at 9 years would suggest that the incubation period would be >18 years. Other prion studies of cynomolgus macaques reported clinical disease within 2–3 years after inoculation with variant Creutzfeldt-Jakob disease agents (*9*), 3 years after inoculation with bovine spongiform encephalopathy agents (*10*,*11*), and 5 years after inoculation with sporadic Creutzfeldt-Jakob disease agents (*9*,*12*). In contrast, our findings indicate that CWD is unlikely to develop in cynomolgus macaques.

The cause of susceptibility to CWD agents in squirrel monkeys and resistance to them in cynomolgus macaques is uncertain. *Prnp/PRNP* gene sequence variation has been linked to disease susceptibility (*8*), and differences in the *PRNP* genes of cynomolgus macaques and the genes of squirrel monkeys could play a major role. Comparison of *PRNP* sequences among cynomolgus macaques and squirrel monkeys showed differences exist at 5 codons (56, 100, 108, 159, and 182) (*7*). It is not clear which difference or combination of changes might confer protection to cynomolgus macaques, or if resistance is caused by other factors. Of the 5 codon differences described above, those of cynomolgus macaques and humans are identical at positions 56, 159, and 182.

Two SM-CWD brain samples were inoculated into squirrel monkeys and cynomolgus macaques to verify that SM-CWD was infectious, test for further adaptation, and to see if SM-CWD was infectious to a broader range of nonhuman primates. Two squirrel monkeys inoculated intracerebrally with SM-CWD brain homogenates (SMP2-CWD) were euthanized at 23–24 mpi (Table 1). These incubation periods decreased by >11 months compared with that of the donor squirrel monkey. Neurologic signs in the 2 SMP2-CWD were more pronounced than observed during the first passage; however, weight loss was reduced. Neuropathologic examination and Western blot for PrPres confirmed TSE in both squirrel monkeys. In contrast to SM-CWD infections, the SMP2-CWD-infected brains had spongiform lesions and PrPres deposition in the occipital lobe (Figure 2, panels A, B). Biochemical comparison of glycoform patterns among CWD, SM-CWD, and SMP2-CWD were made by using 3 different anti-PrP antibodies (L42, 6H4, and 3F4) (Technical Appendix).

Table 2. Cynomolgus macaques inoculated with CWD or squirrel monkey–adapted CWD agents*

Disease incidence†	Inoculum‡	Route of inoculation	Titer inoculated§	Screening mpi¶	Current mpi
0/6	MD-1, Elk-1, WTD-1	Intracerebral	3.2×10^5–2.5×10^6	49, 79, 88, 94	124
0/8#	MD-1, Elk-1, WTD-1	Oral	2.5×10^8–2×10^9	97,106,106	124
0/2	SM-CWD	Intracerebral	NA	NA	72
0/1	Normal elk	Intracerebral	NA	96	NA

*An early version of some of these data are shown in Table 3 of (*7*). CWD, chronic wasting disease; mpi, months post-inoculation; MD, mule deer; WTD, white-tailed deer; SM, squirrel monkey; NA, not applicable.
†Number of monkeys in which prion disease developed over number inoculated.
‡Several different inocula were used for this study. Each individual animal was inoculated with 1 inoculum. Detailed descriptions can be found in (*7*).
§Infectivity titers were determined by using endpoint dilution titer in transgenic mice expressing deer prion protein (PrPres) and are listed as 50% infectious dose per gram of brain.
¶Several monkeys were euthanized during the course of the experiment for conditions unrelated to prion infection such as diabetes, neoplasia, hypocalcemia, and behavioral issues. Brain, spleen, and lymph nodes from these animals were screened for PrPres by using Western blot and immunohistochemical methods. No PrPres-positive tissues were detected.
#One monkey from the original oral inoculation group was euthanized at 1 mpi because of a colonic torsion and has been removed from this group.

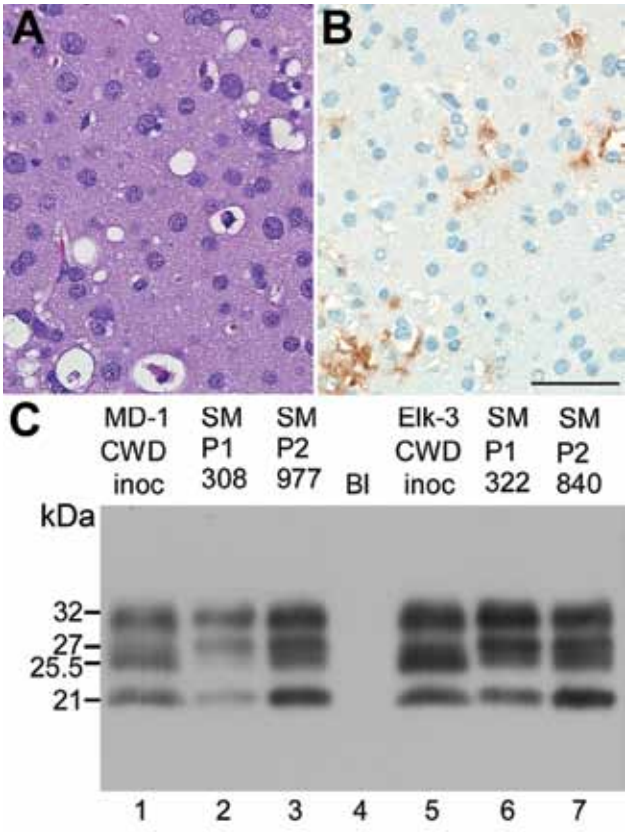


Figure 2. Neuropathologic features and immunoblot results of second-passage squirrel monkeys that had chronic wasting disease (CWD). Scale bar represents 50 μM and is applicable to panels A and B. Panels A and B show neuropathologic changes in the occipital lobe of SMP2-CWD monkey 977, which was euthanized at 24 months postinoculation. A) Hematoxylin and eosin staining show prominent spongiform changes. B) Immunohistochemical staining for disease-associated prion protein (PrPres) (brown) with anti-PrP antibody D13. C) Results of Western blot for PrPres in brain tissue of cervids and its respective first and second passage in squirrel monkeys. MD-1 was used to infect SM308, and SM308 was used to infect SM977. Lanes 1, 2, 5, and 6, 0.6 mg brain equivalents. Lanes 3 and 7, 0.36-mg brain equivalents to give similar signal intensities to the other samples. Lane 4, blank (Bl). Apparent molecular weights (in kDa) are provided on the left side of panel C. Immunoblot was probed with anti-PrP antibody L42. When comparing the 2 central bands, cervid CWD had a more intense band at 25.5 kDa; SM-CWD (nos. 308 and 322) and SM2-CWD (nos. 977 and 840) were more intense at 27 kDa.

In all cases, SM2-CWD had a greater proportion of unglycosylated PrPres and a lower proportion of double glycosylated PrPres than did SM-CWD (Figure 2, panel C). The decreased time of manifestation of disease, differences in glycoform patterns, and distribution of PrPres in brain tissue suggested that the CWD agent was still adapting within the squirrel monkey. However, similar to CWD, SM-CWD had not caused prion disease in cynomolgus macaques by 72 mpi (Table 2).

Conclusion

Our studies have shown that squirrel monkeys, but not cynomolgus macaques, were susceptible to CWD. Although these nonhuman primates are not exact models of human susceptibility, they support the data from transgenic mouse studies (*3*–*6*), in vitro experiments (*13*), and epidemiologic evidence (*14*,*15*) that suggest humans are at a low risk of contracting CWD. Nevertheless, it remains sensible to minimize exposure to tissues potentially contaminated with the CWD agent.

Acknowledgments

We thank Byron Caughey, Kim Hasenkrug, and James Carroll for critical review of the manuscript; Nancy Kurtz, Lori Lubke, and Dan Long for assistance with histology preparation; Don Gardner and Dana Scott for necropsy assistance and lesion interpretation; Ed Schreckendgust, Rocky Rivera, Michael Wagner, Leslie Trail, and Richard Cole for animal husbandry; Michael Parnell, Douglas Brining, and RMVB staff for assistance with nonhuman primate inoculations and health care; and Mike Miller, Terry Kreeger, Jean Jewell, and Lynn Creekmore for CWD-agent positive and negative cervid tissues.

This research was supported by the Intramural Research Program of the NIH, NIAID.

Dr Race is a staff scientist in the Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases. His research interests are infectious diseases of humans and animals.

References

1. Saunders SE, Bartelt-Hunt SL, Bartz JC. Occurrence, transmission, and zoonotic potential of chronic wasting disease. Emerg Infect Dis. 2012;18:369–76. http://dx.doi.org/10.3201/eid1803.110685
2. Gilch S, Chitoor N, Taguchi Y, Stuart M, Jewell JE, Schatzl HM. Chronic wasting disease. Top Curr Chem. 2011;305:51–77. http://dx.doi.org/10.1007/128_2011_159
3. 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. http://dx.doi.org/10.1523/JNEUROSCI.2467-05.2005
4. Tamgüney 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. http://dx.doi.org/10.1128/JVI.00098-06
5. Sandberg MK, Al-Doujaily H, Sigurdson CJ, Glatzel M, O'Malley C, Powell C, et al. Chronic wasting disease prions are not transmissible to transgenic mice overexpressing human prion protein. J Gen Virol. 2010;91:2651–7. http://dx.doi.org/10.1099/vir.0.024380-0
6. Wilson R, Plinston C, Hunter N, Casalone C, Corona C, Tagliavini F, et al. Chronic wasting disease and atypical forms of bovine spongiform encephalopathy and scrapie are not transmissible to mice expressing wild-type levels of human prion protein. J Gen Virol. 2012;93:1624–9. http://dx.doi.org/10.1099/vir.0.042507-0
7. Race B, Meade-White KD, Miller MW, Barbian KD, Rubenstein R, LaFauci G, et al. Susceptibilities of nonhuman primates to chronic wasting disease. Emerg Infect Dis. 2009;15:1366–76. http://dx.doi.org/10.3201/eid1509.090253

8. Lloyd S, Mead S, Collinge J. Genetics of prion disease. Top Curr Chem. 2011;305:1–22. http://dx.doi.org/10.1007/128_2011_157
9. 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. http://dx.doi.org/10.1128/JVI.79.22.14339-14345.2005
10. Lasmézas CI, Deslys JP, Demaimay R, Adjou KT, Lamoury F, Dormont D, et al. BSE transmission to macaques. Nature. 1996;381:743–4. http://dx.doi.org/10.1038/381743a0
11. Ono F, Terao K, Tase N, Hiyaoka A, Ohyama A, Tezuka Y, et al. Experimental transmission of bovine spongiform encephalopathy (BSE) to cynomolgus macaques, a non-human primate. Jpn J Infect Dis. 2011;64:50–4.
12. 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. http://dx.doi.org/10.1002/ana.410350504
13. 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. http://dx.doi.org/10.1093/emboj/19.17.4425
14. 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. http://dx.doi.org/10.3201/eid1006.031082
15. 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. http://dx.doi.org/10.3201/eid1210.060019

Address for correspondence: Brent Race, Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, 903 South 4th St, Hamilton, MT 59840, USA; email: raceb@niaid.nih.gov

Rickettsia spp. in Seabird Ticks from Western Indian Ocean Islands, 2011–2012

Muriel Dietrich, Camille Lebarbenchon, Audrey Jaeger, Céline Le Rouzic, Matthieu Bastien, Erwan Lagadec, Karen D. McCoy, Hervé Pascalis, Matthieu Le Corre, Koussay Dellagi, and Pablo Tortosa

We found a diversity of *Rickettsia* spp. in seabird ticks from 6 tropical islands. The bacteria showed strong host specificity and sequence similarity with strains in other regions. Seabird ticks may be key reservoirs for pathogenic *Rickettsia* spp., and bird hosts may have a role in dispersing ticks and tick-associated infectious agents over large distances.

Rickettsia infections have been explored in many tick species parasitizing a wide variety of animal hosts (*1*). *Rickettsia africae*, the agent of African tick-bite fever, has been detected in the seabird tick *Amblyomma loculosum* (*2*), suggesting a role for seabirds in the maintenance, transmission, and large-scale dispersal of emerging *Rickettsia* spp.

In tropical regions, 2 common and abundant tick species are associated with seabird colonies: the hard tick, *A. loculosum*, and the soft tick, *Carios capensis* (*3*). *Rickettsia* spp. have been documented in these tick species from different areas, although only 1 report is available for tropical areas (*2*). *R. hoogstraalii, R. felis,* and an undescribed species (*Rickettsia* sp. scc49) were detected in *C. capensis* ticks in South Carolina, USA (*4*,*5*). *R. hoogstraalii* was also reported in *C. capensis* ticks from Japan (*6*), raising questions regarding the host and geographic range of *Rickettsia* spp. in seabird ticks.

Author affiliations: Centre de Recherche et de Veille sur les Maladies Émergentes dans l'Océan Indien, Sainte Clotilde, Réunion Island, France (M. Dietrich, C. Lebarbenchon, C. Le Rouzic, M. Bastien, E. Lagadec, H. Pascalis, K. Dellagi, P. Tortosa); Université de La Réunion, Saint Denis, Réunion Island, France (M. Dietrich, C. Lebarbenchon, P. Tortosa, A. Jaeger, C. Le Rouzic, M. Bastien, M. Le Corre); Institut de Recherche pour le Développement, Sainte Clotilde (E. Lagadec, H. Pascalis, K. Dellagi); and Maladies Infectieuses et Vecteurs: Ecologie, Génétique, Evolution et Contrôle, Montpellier, France (K.D. McCoy)

DOI: http://dx.doi.org/10.3201/eid2005.131088

To better understand the role of seabirds and seabird ticks as reservoirs and dispersers of pathogens, we investigated *Rickettsia* spp. infections in *A. loculosum* and *C. capensis* ticks. These 2 tick species are commonly found on remote tropical islands of the western Indian Ocean. Using molecular detection techniques, we examined variations in detection rates and estimated the genetic diversity of *Rickettsia* spp. between islands and between tick species.

The Study

During 2011 and 2012, we collected ticks from seabird colonies on 6 islands of the western Indian Ocean: Aride, Bird, Europa, Juan de Nova, Tromelin, and Réunion Islands (Figure 1; Table). Sample collection on Réunion, Juan de Nova, Tromelin, and Europa Islands was conducted under the approval of the Direction de l'Environnement, de l'Aménagement et du Logement, the Conservatoire du Littoral–Antenne Océan Indien, and the Terres Australes and Antarctiques Françaises. Sample collection on Aride and Bird Islands and their export to Réunion Island were performed with the approval of the Seychelles Bureau of Standards and the Ministry of Environment.

All ticks were morphologically identified as *A. loculosum* or *C. capensis* by using standard taxonomic keys (Figure 2). Ticks were individually washed in distilled water and homogenized in Dulbecco modified essential medium or Buffer AVL (QIAGEN, Valencia, CA, USA). Total nucleic acids were extracted by using the EZ1 Viral

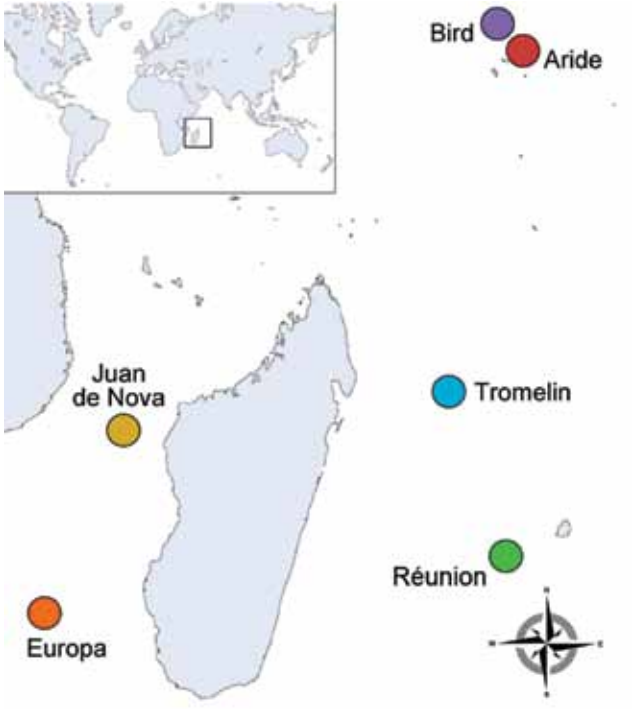


Figure 1. Location of western Indian Ocean islands where tick sampling was conducted among seabird colonies during 2011–2012.

Table. Species and numbers of ticks collected during a study of the distribution and host specificity of *Rickettsia* spp. in seabird ticks on 6 remote islands in the western Indian Ocean, 2011–2012

Island, host species	Tick species	No. ticks detected	Detection rate, %, *Rickettsia* spp. (±95% CI)
Aride			
Puffinus pacificus, Onychoprion fuscatus	*Amblyomma loculosum*	31	45 (18)
Bird			
O. fuscatus	*Carios capensis*	13	8 (15)
	A. loculosum	41	37 (1)
Europa			
O. fuscatus	*C. capensis*	42	74 (1)
Juan de Nova			
O. fuscatus	*C. capensis*	43	16 (1)
Réunion			
P. pacificus	*C. capensis*	39	59 (1)
	A. loculosum	72	46 (12)
Tromelin			
Sula sula, S. dactylatra	*C. capensis*	23	26 (18)
	A. loculosum	14	93 (13)
Total		318	45 (5)

mini kit v2 and the BioRobot EZ1 System or the QIAmp Viral RNA Mini Kit (all from QIAGEN). We used a commercial cDNA kit (Promega, Madison, WI, USA) to generate cDNAs by reverse transcription. cDNA and PCR, with primers selecting for a 1,064-bp fragment of the citrate synthase–encoding gene (*gltA*), were used to detect *Rickettsia* spp. (*7*). Sequencing of this fragment enabled putative identification of the detected rickettsiae, although complete genotyping is required to fully characterize them (*8*).

Overall, 143 of the 318 ticks tested were positive for *Rickettsia* spp., corresponding to a global detection rate (± 95% CI) of 45% (± 5%) (Table); detection from *A. loculosum* (47% ± 8%) and *C. capensis* (43% ± 8%) ticks was similar ($\chi^2 = 0.79$, df = 1, p = 0.37). However, detection rates differed significantly among the islands ($\chi^2 = 37.96$, df = 5, p<0.001). For example, the detection rate was lower in *C. capensis* ticks on Bird (8% ± 15%) and Juan de Nova (16% ± 11%) Islands compared with other islands. The detection rate in *A. loculosum* ticks on Réunion Island was particularly high (93% ± 11%) compared with that on other islands.

Partial sequencing was performed on the *Rickettsia* spp. *gltA* gene from 60 ticks, including ticks from each island and both species. Phylogenetic analyses revealed 7 haplotypes clustering in 4 well-supported clades; some of the haplotypes corresponded to previously described species (Figure 3). No association was found between *Rickettsia* spp. and collection location (island), but strong host (tick) specificity was observed (Figure 3). All *A. loculosum* ticks were infected with a *Rickettsia* strain showing 100% nt identity with the *R. africae* strain infecting *A. loculosum* ticks in New Caledonia and with the ESF-5 strain isolated from the cattle tick, *A. variegatum*, in Ethiopia (GenBank accession no. CP001612.1). *Rickettsia* spp. in *C. capensis* ticks had 3 well-supported genetic clusters and, thus, were more diverse. The most frequent *Rickettsia* sp. in these ticks (64% of the sequences) was a probable strain of *R. hoogstraalii.* Other haplotypes corresponded to an unknown *Rickettsia sp.* that was closely related to *R. hoogstraalii* and to a probable strain of *R. bellii* (28% and 8% of the sequences, respectively). The haplotype of *R. bellii* was detected only on Réunion Island in 2 *C. capensis* ticks collected within the same nest of the wedge-tailed shearwater (*Puffinus pacificus*).

Figure 2. *Amblyomma loculosum* (left) and *Carios capensis* (right) ticks from seabird colonies on western Indian Ocean islands.

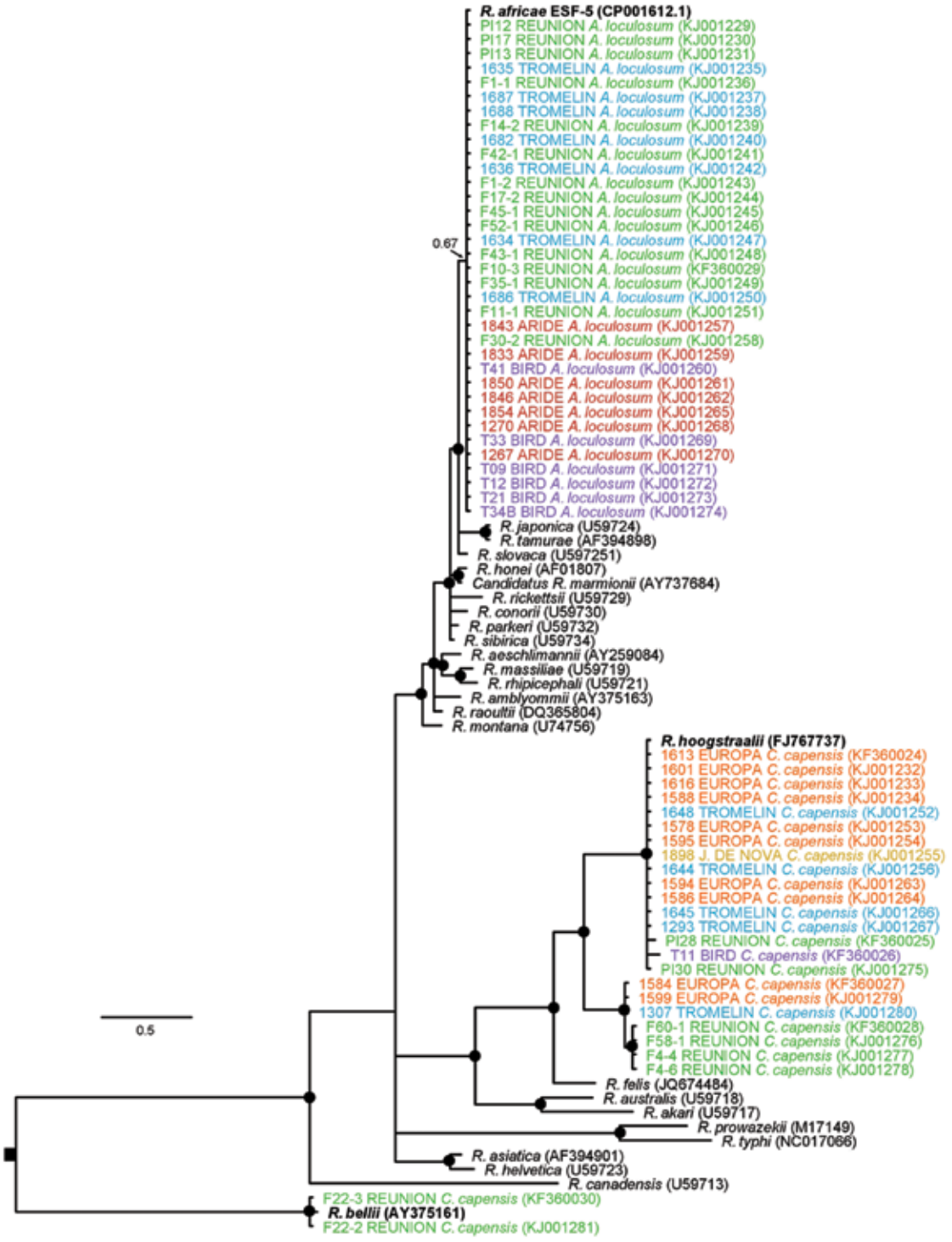


Figure 3. Maximum clade credibility tree for *Rickettsia* spp. detected in seabird ticks (*Amblyomma loculosum* and *Carios capensis*) of the western Indian Ocean as determined on the basis of a 913-bp fragment of the *Rickettsia gltA* gene. The nucleotide substitution model was selected by using the jModelTest 2.1.2 tool (https://code.google.com/p/jmodeltest2/), and Bayesian analyses were performed using MrBayes 3.1.2 (http://mrbayes.sourceforge.net/), with chain lengths of 2 million generations sampled every 1,000 generations. Black dots indicate Bayesian posterior probabilities >0.7. Taxa names are represented by the identification of the sample, the sampling site, and the tick species. GenBank accession numbers are indicated in parentheses. The sequences generated in this study are color-coded according to the geographic origin of samples (see Figure 1) and are accessible in GenBank (accession nos. KF360024–KF360030 and KJ001229–KJ001281). Scale bar indicates nucleotide substitutions per site.

Conclusions

We provide evidence of *rickettsia* infection in seabird ticks from 6 islands in the western Indian Ocean. Although the tick infection rate varied among the islands, *Rickettsia* spp. were detected in all studied seabird populations and overall *Rickettsia* infection rates were comparable to those reported for ticks parasitizing terrestrial hosts (*9*).

Phylogenetic analyses revealed a diversity of *Rickettsia* spp. showing strong host specificity. The most frequently detected *Rickettsia* sp. was *R. africae*, the causative agent of African tick-bite fever. *R. africae* was found only in the hard tick, *A. loculosum*. These findings support the hypothesis that *R. africae* is well adapted to this tick host, which may act as a major reservoir for the pathogen (*8*). Moreover, the similarity of *R. africae* sequences from our study with those of strains found in other parts of the world indicates that host shifts may be frequent for this bacterium, facilitating its spread in different tick populations. A better understanding of exchanges between wild and domestic fauna and the possible emergence of *R. africae* at regional levels will require further investigations of the link between *R. africae* in seabird ticks and in cattle ticks (*10*).

We detected 2, possibly 3, *Rickettsia* spp. in *C. capensis* ticks; this genetic diversity was higher than that detected in *A. loculosum* ticks. In accordance with previous descriptions (*4*,*6*), *R. hoogstraalii* was the most common *Rickettsia* species infecting *C. capensis* ticks, confirming that this tick species likely represents a major host reservoir for this bacterium. An unclassified rickettsial lineage, genetically related to *R. hoogstraalii*, was also identified in *C. capensis* from Réunion, Europa, and Tromelin Islands, showing that 2 distinct *Rickettsia* spp. cocirculate in these tick populations. Additional sampling and further genetic characterization (*8*) are required to better describe the distribution of this lineage and its relationships to *R. hoogstraalii*.

R. bellii has mainly been documented in the Americas, but recent studies have detected the presence of closely related *R. bellii* strains in Australia (*11*) and Europe (*12*). Although we detected *R. bellii* in only 2 ticks on 1 island, our study findings show the potential for broad distribution of this bacterium because of its large arthropod host range (*13*). The *R. bellii* genome has been completely sequenced (*13*), but current knowledge on its epidemiology and pathogenicity in humans remains to be evaluated. In addition, our study was restricted to analysis of the *gltA* gene, so additional genotyping studies are needed to fully characterize the *Rickettsia* spp. that we identified (*8*).

The presence of *Rickettsia* spp. on remote islands indicates a possible role of bird hosts in the dispersal of ticks and their associated infectious agents over large distances (*14*,*15*). An understanding of the historic colonization and current population structure of *A. loculosum* and *C. capensis* ticks, together with knowledge of bird migratory patterns, is required to properly assess the risk for emergence of *Rickettsia* spp. on these islands.

Acknowledgments

We thank Sébastien Lefort, Aurélien Prudor, Chris Feare, Christine Larose, Gérard Rocamora, and Sarah Temmam for assistance with tick sampling and molecular work.

This work was supported by the Fond Européen de Développement Régional Pathogenes associés à la Faune Sauvage Océan Indien (Programme Opérationnel de Coopération Territoriale 2007–2013; no. 31189) and by the Centre National de la Recherche Scientifique–Institut National de l'Ecologie et de l'Environnement/Terres Australes et Antarctiques Françaises (Iles Eparses PathOrnithoTiques project). Postdoctoral fellowships (to M.D., C.L., and A.J.) were funded by RUN-Emerge European project funded by European Commission under FP7 program. C.LeR. and M.B. were supported by a training program fellowship from the Federation Environnement–Biodiversité–Santé.

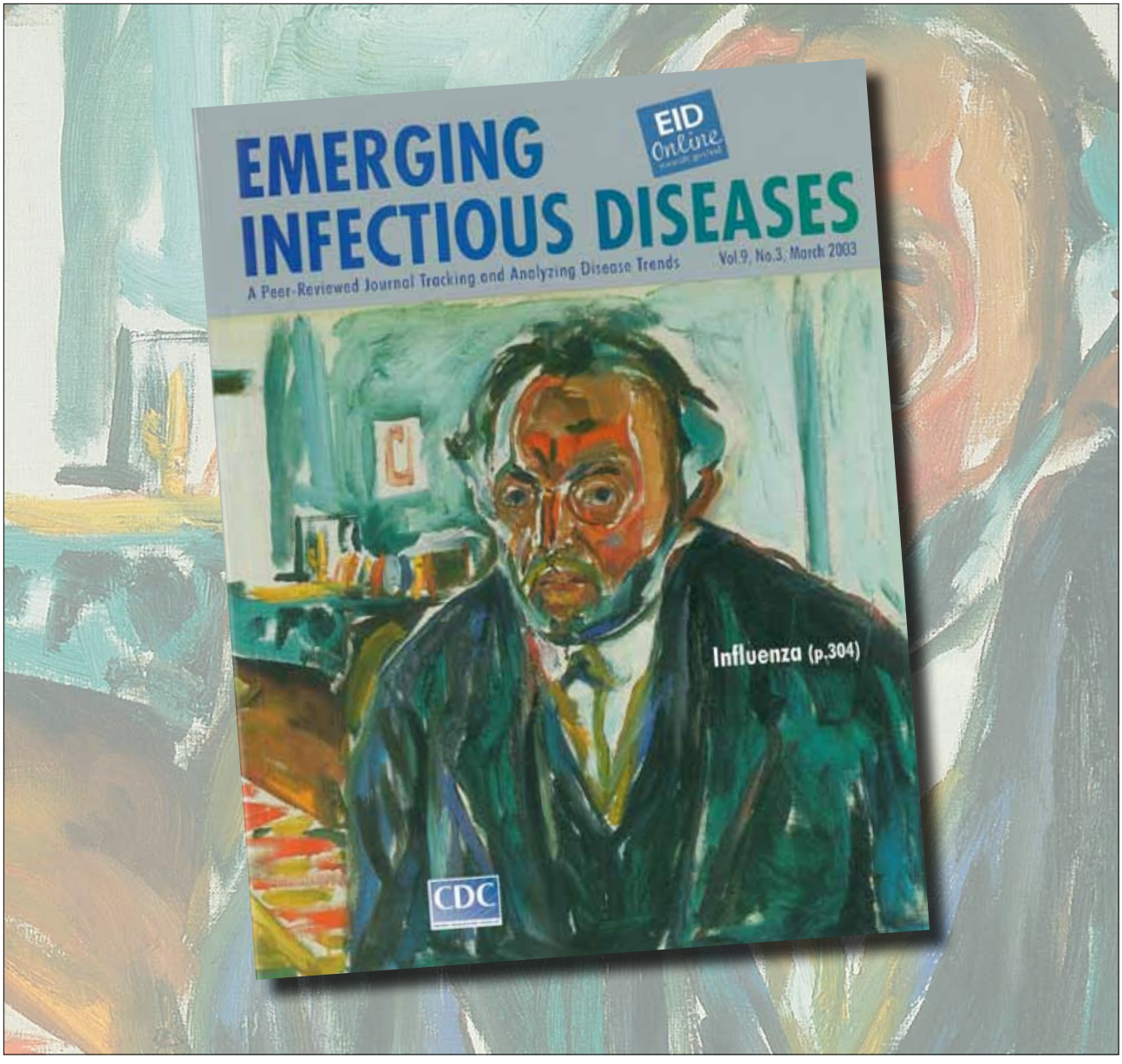
Dr Dietrich is a postdoctoral research associate in the Center for Research on Emerging Infectious Diseases in the Indian Ocean on Réunion Island. She is interested in parasite biodiversity and the evolutionary ecology of host–parasite interactions.

References

1. Parola P, Paddok CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev. 2005;18:719–56. http://dx.doi.org/10.1128/CMR.18.4.719-756.2005
2. Eldin C, Mediannikov O, Davoust B, Cabre O, Barré N, Raoult D, et al. Emergence of *Rickettsia africae*, Oceania. Emerg Infect Dis. 2011;17:100–2. http://dx.doi.org/10.3201/eid1701.101081
3. Dietrich M, Gómez-Díaz E, McCoy KD. Worldwide distribution and diversity of seabird ticks: implications for the ecology and epidemiology of tick-borne pathogens. Vector Borne Zoonotic Dis. 2011;11:453–70. http://dx.doi.org/10.1089/vbz.2010.0009
4. Reeves WK, Loftis AD, Sanders F, Spinks MD, Wills W, Denison AM, et al. *Borrelia, Coxiella*, and *Rickettsia* in *Carios capensis* (Acari: Argasidae) from a brown pelican (*Pelecanus occidentalis*) rookery in South Carolina, USA. Exp Appl Acarol. 2006;39:321–9. http://dx.doi.org/10.1007/s10493-006-9012-7
5. Mattila JT, Burkhardt N, Hutcheson J, Munderloh UG, Kurtti TJ. Isolation of cell lines and a rickettsial endosymbiont from the soft tick *Carios capensis* (Acari: Argasidae: Ornithodorinae). J Med Entomol. 2007;44:1091–101. http://dx.doi.org/10.1603/0022-2585(2007)44[1091:IOCLAA]2.0.CO;2
6. 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. http://dx.doi.org/10.1111/j.1348-0421.2006.tb03807.x
7. Davis MJ, Ying Z, Brunner BR, Pantoja A, Ferwerda FH. Rickettsial relative associated with papaya bunchy top disease. Curr Microbiol. 1998;36:80–4. http://dx.doi.org/10.1007/s002849900283
8. Fournier PE, Dumler JS, Greub G, Zhang J, Wu Y, Raoult D. Gene sequence-based criteria for identification of new *Rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. J Clin Microbiol. 2003;41:5456–65. http://dx.doi.org/10.1128/JCM.41.12.5456-5465.2003

9. Mediannikov O, Trape JF, Diatta G, Parola P, Fournier PE, Raoult D. *Rickettsia africae*, western Africa. Emerg Infect Dis. 2010;16: 571–3. http://dx.doi.org/10.3201/eid1603.090346
10. Parola P, Barre N. *Rickettsia africae*, agent de la fièvre à tique africaine: un pathogène émergent dans les Antilles et l'île de la Réunion. Bull Soc Pathol Exot. 2004;97:193–8.
11. Vilcins IME, Old JM, Deane E. Molecular detection of *Rickettsia, Coxiella* and *Rickettsiella* DNA in three native Australian tick species. Exp Appl Acarol. 2009;49:229–42. http://dx.doi.org/10.1007/s10493-009-9260-4
12. Sprong H, Wielinga PR, Fonville M, Reusken C, Brandenburg AH, Borgsteede F, et al. *Ixodes ricinus* ticks are reservoir hosts for *Rickettsia helvetica* and potentially carry flea-borne *Rickettsia* species. Parasit Vectors. 2009;2:41–7. http://dx.doi.org/10.1186/1756-3305-2-41
13. Ogata H, La Scola B, Audic S, Renesto P, Blanc G, Robert C, et al. Genome sequence of *Rickettsia bellii* illuminates the role of amoebae in gene exchanges between intracellular pathogens. PLoS Genet. 2006;2:e76. http://dx.doi.org/10.1371/journal.pgen.0020076
14. Palomar AM, Santibáñez P, Mazuelas D, Roncero L, Santibáñez S, Portillo A, et al. Role of birds in dispersal of etiologic agents of tick-borne zoonoses, Spain, 2009. Emerg Infect Dis. 2012;18:1188–91. http://dx.doi.org/10.3201/eid1807.111777
15. Palomar AM, Portillo A, Santibáñez P, Mazuelas D, Arizaga J, Crespo A, et al. Crimean–Congo hemorrhagic fever virus in ticks from migratory birds, Morocco. Emerg Infect Dis. 2013;19:260–3. http://dx.doi.org/10.3201/eid1902.121193

Address for correspondence: Muriel Dietrich, Centre de Recherche et de Veille sur les maladies émergentes dans l'Océan Indien (CRVOI), Plateforme de Recherche CYROI, 2 rue Maxime Rivière, BP 80005, 97491 Sainte Clotilde, Réunion Island; email: muriel.dietrich@gmail.com



Influenza A Subtype H3 Viruses in Feral Swine, United States, 2011–2012

Zhixin Feng, John A. Baroch, Li-Ping Long, Yifei Xu, Frederick L. Cunningham, Kerri Pedersen, Mark W. Lutman, Brandon S. Schmit, Andrew S. Bowman, Thomas J. DeLiberto, and Xiu-Feng Wan

To determine whether, and to what extent, influenza A subtype H3 viruses were present in feral swine in the United States, we conducted serologic and virologic surveillance during October 2011–September 2012. These animals were periodically exposed to and infected with A(H3N2) viruses, suggesting they may threaten human and animal health.

Swine are proposed as "mixing vessels" to generate novel influenza A viruses (IAVs) by facilitating reassortment among IAVs and providing a potential pathway in which these viruses can move from wild birds to humans (*1*). Subtype H3N2 is one of the most common subtypes in the US domestic swine population, which possibly resulted from spillover of human seasonal A(H3N2) virus (*2*,*3*). Since its introduction in the mid-1990s, A(H3N2) virus has evolved genetically and antigenically in domestic swine. Four genetic groups (so-called clusters I–IV) were identified, and these 4 clusters were also antigenically distinct (*4*). The viruses in cluster IV formed at least 2 antigenic subclusters, H3N2-α and H3N2-β (*5*). Both subclusters are co-circulating in pigs, and subcluster H3N2-β predominated among the isolates obtained from domestic swine at Ohio county fairs during 2010–2011 (*5*). During July and August 2011, two children were infected with novel reassortant H3N2 variant (H3N2v), 1 in Indiana and 1 in Pennsylvania (*6*). This H3N2v virus is antigenically similar to the viruses in subcluster H3N2-β and has the matrix gene of influenza A(H1N1)pdm09 virus. It caused illness in ≈2,055 persons during August 2011–April 2012 (*7*).

The role of feral swine in IAV ecology has not been adequately addressed. Feral swine could be a reservoir of IAVs or, possibly, a spatially dynamic mixing vessel, given their free-ranging habits. Such unrestricted movement provides the potential for exchange of IAVs among subpopulations of feral swine and the opportunity for exposure to different IAVs through contact with a variety of habitats and species. Also, feral swine can live up to 8 years, which provides ample opportunities for reinfection with the same subtype IAVs, especially those with antigenically distinct hemagglutinins. The IAVs can be transmitted bidirectionally between feral and domestic swine because contact between them is not unusual (*8*). Ultimately the IAVs emerging in feral swine potentially could be transmitted to humans.

The United States has ≈4–5 million feral swine (*9*) throughout at least 38 states (*10*). Feral swine are expanding their range because of a lack of natural predators and intentional introductions for hunting. Our goals in this study were to determine through virologic and serologic surveillance whether, and to what extent, subtype H3 IAVs were present in the US feral swine metapopulation.

The Study

We collected 1,983 nasal swab samples from swine during October 2011–September 2012 (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1578-Techapp1.pdf). Matrix gene–based quantitative reverse transcription PCR showed that 9 swabs samples were IAV positive; 1 A(H3N2) feral swine isolate, A/swine/Texas/A01104013/2012(H3N2), was recovered. Phylogenetic analyses showed that all genes of this feral swine isolate are genetically similar to those of A(H3N2v) viruses isolated from humans, and other contemporary subtype H3N2 isolates from swine from county fairs farms. The matrix gene of this feral swine IAV was genetically close to that of A(H1N1)pdm09 and the human A(H3N2v) viruses (online Technical Appendix Figure) (*11*). Similar to other viruses in the antigenic cluster, H3N2-β A/swine/Texas/A01104013/2012(H3N2) had a R189K mutation at the antibody-binding site of the hemagglutinin protein, which caused a recent antigenic drift in subtype H3N2 IAVs (*5*,*12*). The 8 genes of A/swine/Texas/A01104013/2012(H3N2) have a minimal 99.59% nt sequence identity to those of the human subtype H3N2v isolate A/Indiana/10/2011(H3N2).

We also collected 1,989 serum samples from swine in 31 states; these samples were tested by using an IAV-specific ELISA (Figure 1). We identified 182 samples as

Author affiliations: Mississippi State University, Mississippi State, Mississippi, USA (Z. Feng, L.-P. Long, Y. Xu, X.-F. Wan); Jiangsu Academy of Agricultural Sciences, Nanjing, China (Z. Feng); Animal and Plant Health Inspection Service of the US Department of Agriculture, Fort Collins, Colorado, USA (J.A. Baroch, K. Pedersen, M.W. Lutman, B.S. Schmit, T.J. DeLiberto); Animal and Plant Health Inspection Service of the US Department of Agriculture, Mississippi State (F.L. Cunningham); and The Ohio State University, Columbus, Ohio, USA (A.S. Bowman)

DOI: http://dx.doi.org/10.3201/eid2005.131578

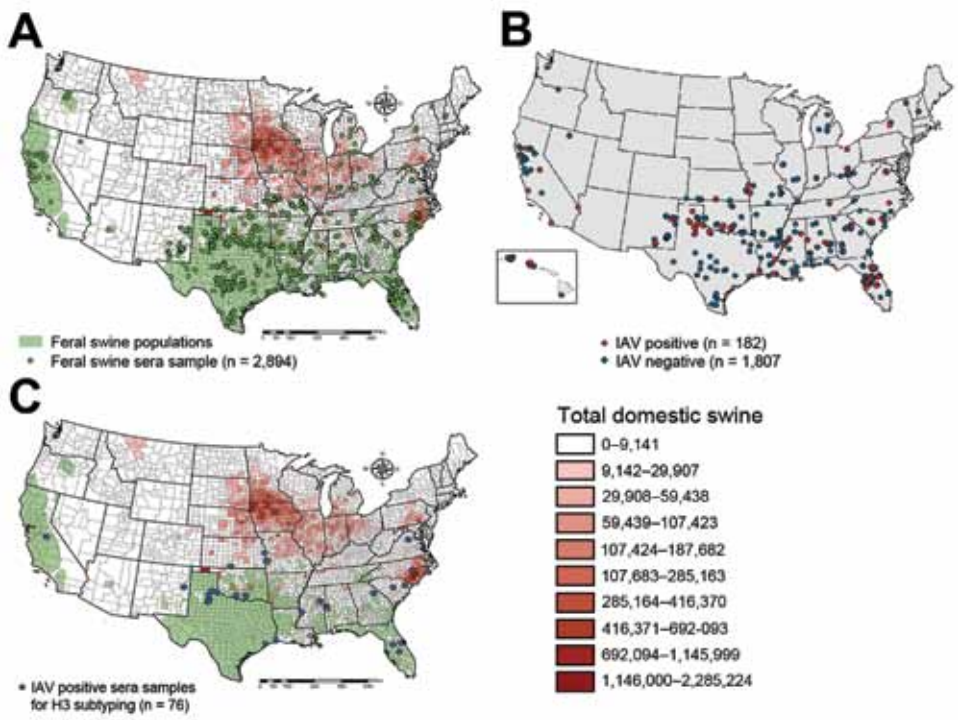


Figure 1. Geographic distributions of seum samples from feral swine, United States, 2011–2012. A) Of 1,989 samples tested by ELISA, 182 were positive (red) and 1,807 were negative (blue). B) The 76 samples (blue) were selected for hemagglutination-inhibition and microneutralization subtyping. C) The distributions of feral swine (green) and domestic swine (orange) were also marked (A and C).

IAV positive, from swine that were broadly distributed over 19 states. The average IAV seropositive rate was 9.15% but it varied by month. The highest positive rate (22.9%) was in June 2012 (Figure 2). Although no clear temporal pattern was found in the IAV seropositive rate, the rate was relatively higher in the summer than in other seasons. One explanation could be that noncommercial swine farmers might give their animals more pasture time during the summer, thereby increasing the chance of contact between domestic and feral swine. In addition, our results showed adult feral swine (≥1 year of age) had the highest rate of IAV positivity (11.1% [of 1,380 animals]), followed by subadults (2 months–1 year of age) (5.1% [of 494 animals]), and juveniles (<2 months of age) (3.8% [of 105 animals]) (age was not determined for 10 animals). (Dentition patterns were used to determine the age of feral swine [*13*].) Female and male pigs were equally as likely to be seropositive (102 [9.6%] of 1,058 vs. 80 [9.7%] of 821).

Of the 182 IAV-positive serum samples, 76 were randomly selected for influenza subtyping. We used hemagglutination-inhibition (HI) and microneutralization (MN) assays for subtyping against A(H1N1)pdm09 virus and 22 H3 IAVs, which represent a wide range of antigenically

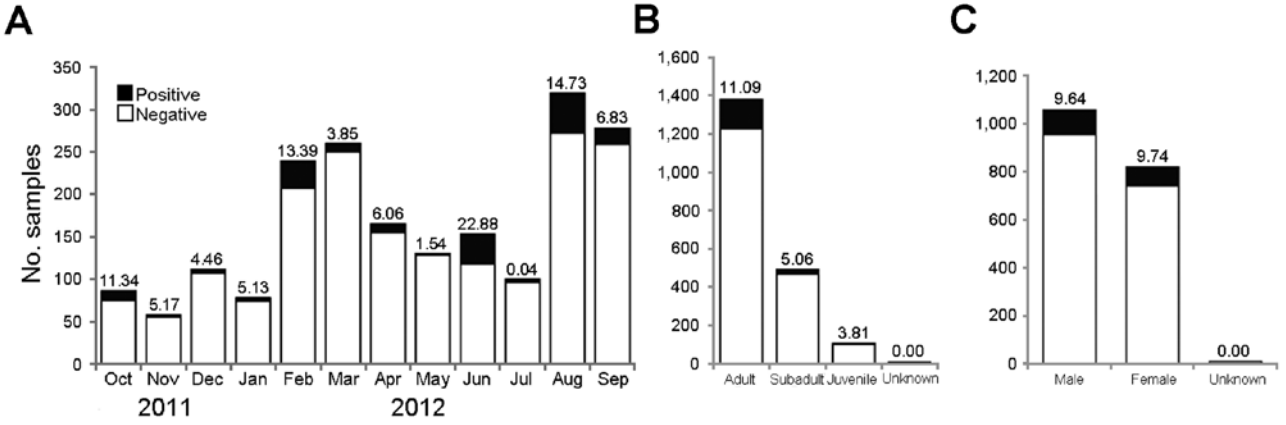


Figure 2. Epidemiologic analyses of feral swine serum samples seropositive for influenza A virus by ELISA, United States, 2011–2012. A) Temporal distribution. B) Distribution of feral pigs, by age. C) Distribution of feral pigs, by sex. The numbers on the bar indicate the influenza A virus–seropositive percentile.

Table. Cross-reactive antibody responses against H3 and influenza A(H1N1)pdm09 virus in 76 influenza-positive serum samples from feral swine, United States, 2011–2012*

Source, virus	Antigenic cluster†	No. (%) seropositive‡ HI	No. (%) seropositive‡ MN	GMT (95% CI)§ HI	GMT (95% CI)§ MN	Overall no. (%) seropositive¶
Feral swine IAV						
A/swine/Texas/A011040013/2012(H3N2)	H3N2-β	36 (47.4)	36 (47.4)	163 (40–640)	259 (40–1,280)	25 (32.9)
Domestic swine IAV						
A/swine/Ohio/11SW64/2009 (H3N2)	H3N2-α	27 (35.5)	30 (39.5)	65 (40–160)	121 (40–1,280)	17 (22.4)
A/swine/Iowa/12627/2009(H3N2)	H3N2-α	30 (39.5)	31 (40.8)	73 (40–320)	183 (40–1,280)	15 (19.7)
A/swine/Ohio/11SW347/2011(H3N2)	H3N2-β	31 (40.8)	31 (40.8)	122 (40–640)	112 (40–1,280)	17 (22.4)
A/swine/Iowa/6368/2012(H3N2)	H3N2-β	26 (34.2)	36 (47.4)	94 (0–640)	240 (40–1,280)	20 (26.3)
Human H3N2v IAV						
A/IA/07/2011		32 (42.1)	31 (40.8)	109 (40–640)	313 (40–1,280)	21 (27.6)
Human seasonal IAV	H3N2-β					
A/Perth/16/2009	H3N2-β	12 (15.8)	5 (6.6)	76 (40–320]	121 (40–1,280)	1 (1.3)
A/Victoria/361/2011	H3N2-β	11 (14.5)	2 (2.6)	141 (40–640)	226 (40–1,280)	0
A/California/7/2009	H3N2-β	5 (6.6)	3 (3.9)	70 (40–640)	80 (40–320)	1 (1.3)

*Influenza detected by ELISA. GMT, geometric mean titer; HI, hemagglutination-inhibition; MN, microneutralization; IAV, influenza A virus.
†The antigenic cluster H3N2-α and H3N2-β were defined in (*5*).
‡Seropositive was defined as the HI or MN titer ≥40.
§Only serum with an HI or MN titer ≥40 are used to calculate the GMT.
¶Overall seroconversion was defined as both HI and MN titer ≥40.

distinct H3 IAVs (online Technical Appendix Table). Serum was defined as seropositive if its titer was ≥40.

HI results showed that 46 (60.5%) of 76 feral swine samples were positive against at least 1 of the 22 H3 IAVs tested, of which A/swine/Texas/A011040013/2012(H3N2) had the highest seropositive rate (47.4%), followed by 4 human A(H3N2v) isolates (Table). MN results were consistent with those of HI. The geometric mean titers for HI and MN against the feral swine isolate were 163 and 259, respectively. The maximum MN titer among the serum and A/swine/Texas/A011040013/2012(H3N2) was 1,280. The seropositive rates of these serum samples varied from 34.2% to 42.1% against the other viruses from subclusters H3N2-α and H3N2-β (Table), but the viruses in subcluster H3N2-β had significantly higher geometric mean titers than did those in H3N2-α ($p<0.001$).

HI results also showed that 12 samples were seropositive against A/Perth/16/2009(H3N2); 11 were seropositive against A/Victoria/361/2011(H3N2), and 5 were seropositive against A/California/7/2009(H1N1). These 3 viruses do not cross-react with the IAVs from subclusters H3N2-α and H3N2-β. Those 5 subtype H1N1-positive samples were also seropositive against subtype H3N2 IAVs, indicating potential previous exposures of these feral swine to both H3 and H1 IAVs.

The HI results demonstrated that only 2 serum samples had a low-level cross-reaction with avian influenza A(H3N2) viruses, and the HI titers for both were 40. This result is consistent with findings in an earlier report (*14*). Further studies are needed to determine whether additional antigenic clusters of H3 IAVs are present in migratory waterfowl. The discrepancies in the cross-reactivity of these serum samples against the IAVs tested in this study suggested that these feral swine had different exposure histories against antigenically diverse IAVs.

Conclusions

Our study demonstrated that subtype H3N2 IAVs are periodically infecting feral swine in the United States. Feral swine are a potential source of IAVs with bidirectional transmission to domestic swine or humans. Detection of an H3N2v-like IAV in the feral swine population demonstrates a potential threat to human health. Continued surveillance is recommended to monitor the distribution and the genomic and antigenic diversities of IAVs in feral swine to better assess the risk.

Acknowledgments

We are grateful to Jianqiang Ye and Brigitte Martin for technical support and discussion and Dena Pruett for editorial assistance. We also thank Michael Shaw providing subtype H3N2v isolates and Kyoung-Jin Yoon for providing swine influenza isolates.

This work was supported by the US Department of Agriculture, Wildlife Services, National Wildlife Research Center (grant no. 13-7428-0961-CA).

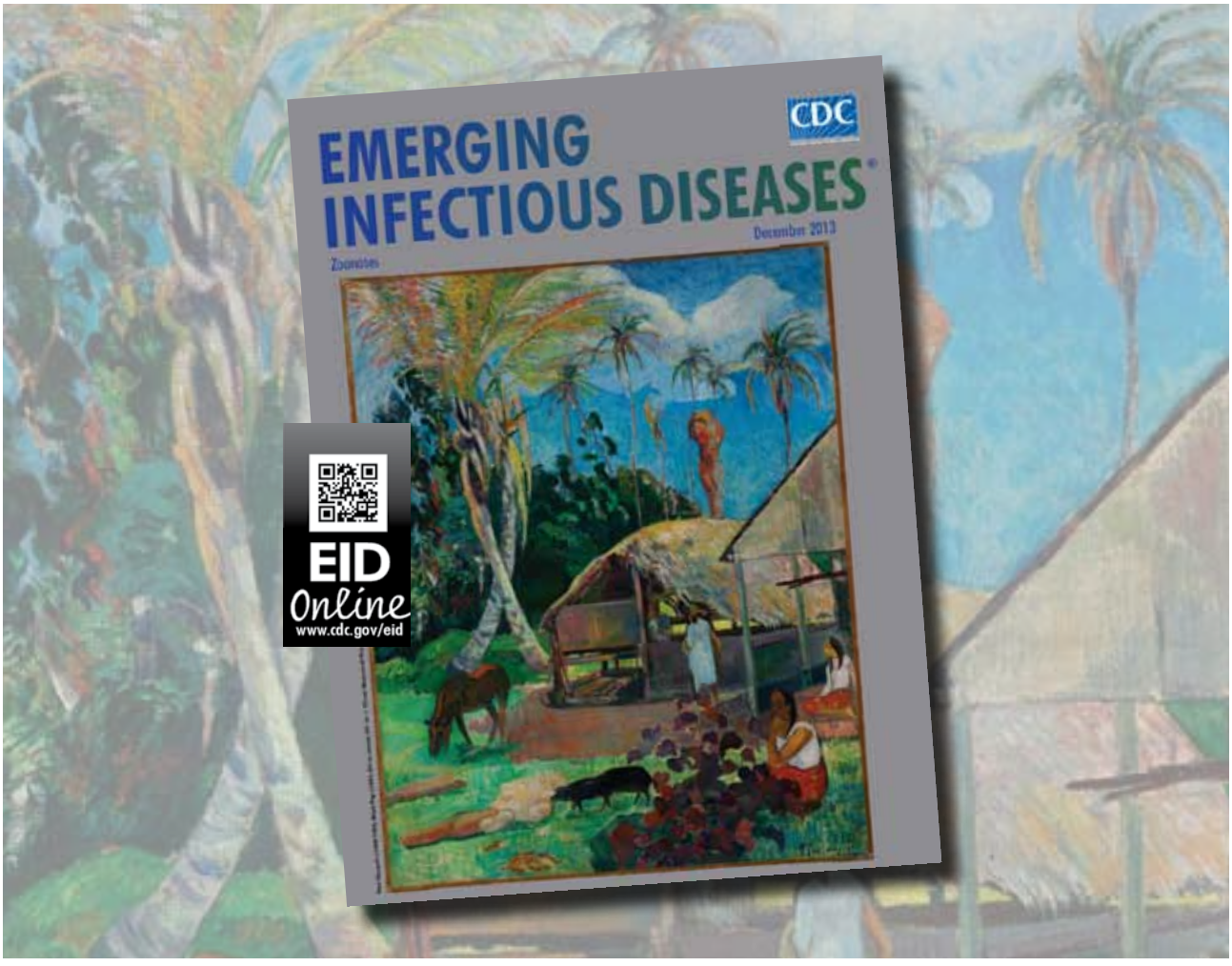
Dr Feng is a visiting scholar at the Influenza Systems Biology Laboratory, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University. His research focuses on swine influenza serology and ecology.

References

1. Hinshaw VS, Webster RG, Easterday BC, Bean WJ Jr. Replication of avian influenza A viruses in mammals. Infect Immun. 1981;34:354–61.
2. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, et al. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol. 1999;73:8851–6.
3. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, et al. Emergence of H3N2 reassortant influenza A viruses in North American pigs. Vet Microbiol. 2000;74:47–58. http://dx.doi.org/10.1016/S0378-1135(00)00165-6

4. Hause BM, Oleson TA, Bey RF, Stine DL, Simonson RR. Antigenic categorization of contemporary H3N2 swine influenza virus isolates using a high-throughput serum neutralization assay. J Vet Diagn Invest. 2010;22:352–9. http://dx.doi.org/10.1177/104063871002200302
5. Feng Z, Gomez J, Bowman AS, Ye J, Long LP, Nelson SW, et al. Antigenic characterization of H3N2 influenza A viruses from Ohio agricultural fairs. J Virol. 2013;87:7655–67. http://dx.doi.org/10.1128/JVI.00804-13
6. Centers for Disease Control and Prevention. Swine-origin influenza A (H3N2) virus infection in two children—Indiana and Pennsylvania, July–August 2011. MMWR Morb Mortal Wkly Rep. 2011;60:1213–5.
7. Biggerstaff M, Reed C, Epperson S, Jhung MA, Gambhir M, Bresee JS, et al. Estimates of the number of human infections with influenza A(H3N2) variant virus, United States, August 2011–April 2012. Clin Infect Dis. 2013;57(Suppl 1):S12–5. http://dx.doi.org/10.1093/cid/cit273
8. Wyckoff AC, Henke SE, Campbell TA, Hewitt DG, VerCauteren KC. Feral swine contact with domestic swine: a serologic survey and assessment of potential for disease transmission. J Wildl Dis. 2009;45:422–9. http://dx.doi.org/10.7589/0090-3558-45.2.422
9. Pimentel D. Environmental and economic costs of vertebrate species invasions into the United States. In: Witmer GW, Pitt WC, Fagerstone KA, editors. Managing vertebrate invasive species: proceedings of an international symposium; 2007 August 7–9, 2007. Fort Collins (CO): USDA/APHIS/National Wildlife Research Center; 2007. p. 1–8.
10. Fogarty EP. National distribution of and stakeholder attitudes toward feral hogs. Mississippi State (MS): Mississippi State University; 2007.
11. Shu B, Garten R, Emery S, Balish A, Cooper L, Sessions W, et al. Genetic analysis and antigenic characterization of swine origin influenza viruses isolated from humans in the United States, 1990–2010. Virology. 2012;422:151–60. http://dx.doi.org/10.1016/j.virol.2011.10.016
12. Ye J, Xu Y, Harris J, Sun H, Bowman AS, Cunningham F, et al. Mutation from arginine to lysine at the position 189 of hemagglutinin contributes to the antigenic drift in H3N2 swine influenza viruses. Virology. 2013;446:225–9. http://dx.doi.org/10.1016/j.virol.2013.08.004
13. Matschke GH. Aging European wild hogs by dentition. J Wildl Manage. 1967;31:109–13. http://dx.doi.org/10.2307/3798365
14. Hall JS, Minnis RB, Campbell TA, Barras S, Deyoung RW, Pabilonia K, et al. Influenza exposure in United States feral swine populations. J Wildl Dis. 2008;44:362–8. http://dx.doi.org/10.7589/0090-3558-44.2.362

Address for correspondence: Xiu-Feng Wan, Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, 240 Wise Center Dr, Mississippi State, MS 39762, USA; email: wan@cvm.msstate.edu



PCR for Detection of Oseltamivir Resistance Mutation in Influenza A(H7N9) Virus

Wei Wang,[1] Zhigang Song,[1] Wencai Guan,[1] Yi Liu, Xiaonan Zhang, Lei Xu, Jianhua Li, Zhenghong Yuan, and Yunwen Hu

Sensitive molecular techniques are needed for rapid detection of the R292K oseltamivir-resistant mutant of influenza A(H7/N9) virus strain to monitor its transmission and guide antiviral treatment. We developed a real-time reverse transcription PCR and single nucleotide polymorphism probes to differentiate this mutant strain in mixed virus populations in human specimens.

An outbreak of human infections with a novel reassortant avian-origin influenza A(H7N9) virus occurred in several provinces of China during March 2013 (*1*), This outbreak caused 137 laboratory-confirmed cases and 45 deaths as of October 2013 (www.who.int/csr/don/2013_10_24a/en/index.html). An unusually high proportion of severe cases and a high case-fatality rate have been observed for patients infected with this virus (*2*).

We reported emergence of an influenza virus with a mutation in the neuraminidase (NA) gene (R292K) and its association with severe clinical outcome in infected persons (*3*). Studies have shown that the NA R292K mutation can cause a high level of resistance to oseltamivir in influenza A(H7N9) virus (*4,5*). Thus, sensitive molecular techniques are needed for rapid detection of influenza virus with this mutation to monitor its circulation and transmission and guide antiviral treatment. In this study, we developed a single-nucleotide polymorphism (SNP) real-time reverse transcription PCR (RT-PCR) to differentiate NA 292K mutant virus from R292 wild-type virus in clinical samples.

The Study

The NA R292K assay has 2 reactions with 1 pair of primers. One reaction contained a FAM-labeled SNP probe specific for the 292K mutant strain and a second reaction contained a VIC-labeled probe specific for the R292 wild-type strain (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1364-Techapp1.pdf).

To assess the sensitivity of the assay, we constructed 2 plasmids that contained R292 wild-type virus or 292K mutant virus, respectively. Fragments of the NA gene inserted into the plasmids were amplified from nasopharyngeal swab specimens from 2 patients infected with influenza A(H7N9) virus and confirmed by using Sanger sequencing (online Technical Appendix). The 2 plasmids were serially diluted 10-fold (10^1–10^{11} copies) in sterile water and used to test the assay. The linear range of sensitivity was 10^2–10^8 copies. The lower limit of detection was 100 copies/reaction (3/3 reactions detected) for wild-type and mutant virus (Figure). However, the sensitivity of the duplex reaction containing both probes was 100-fold lower than that of each separate reaction.

Of 35 respiratory samples tested, 6 were infected with influenza A(H3N2), 2 with influenza A(H1N1) virus, 6 with influenza A(H1N1)pdm09 virus, 4 with parainfluenza virus, 4 with human rhinovirus, 4 with human coronavirus, 5 with influenza B virus, and 4 with respiratory syncytial virus. In addition, 6 other respiratory samples were virus negative. Cross-amplification was not observed during sample testing. Thus, the assay is highly specific for detecting the mutant NA gene of influenza A(H7N9) virus.

To test the performance of the assay when 292K mutant and R292 wild-type viruses were present in 1 sample, a series of mixtures containing the 292K plasmid and the R292 plasmid at copy numbers of 10^4 copies/reaction were prepared at the following ratios of mutant virus to wild-type virus: 2:98, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 95:5, and 98:2. The $\Delta C_t K - R$ of the mixture a ratio of 50:50 was used as the assay-specific normalization value in determination of the percentage of 292K mutant in mixed population as described by Liu et al. (*6*). The assay detected the 292K mutant in the mixture at a proportion of 2% of the 10^4 copies/reaction, and correct estimation of its proportion ranged from 10% to 98% (online Technical Appendix).

To validate the assay with clinical samples, we tested 11 paired nasopharyngeal swab specimens and sputum specimens obtained from 9 patients infected with influenza A(H7N9) virus who had various disease outcomes (Table). The time of sampling (mean 12.6 days, range 7–20 days) was at the end of treatment with an NA inhibitor (oseltamivir or peramivir) or afterwards. Eleven of 22 samples were positive for influenza A(H7N9) virus by a quantitative real-time RT-PCR described in a previous study (*3*). Seven of 11 samples had positive results in the R292K assay:

Author affiliation: Shanghai Public Health Clinical Center, Shanghai, China

DOI: http://dx.doi.org/10.3201/eid2005.131364

[1]These authors contributed equally to this article.

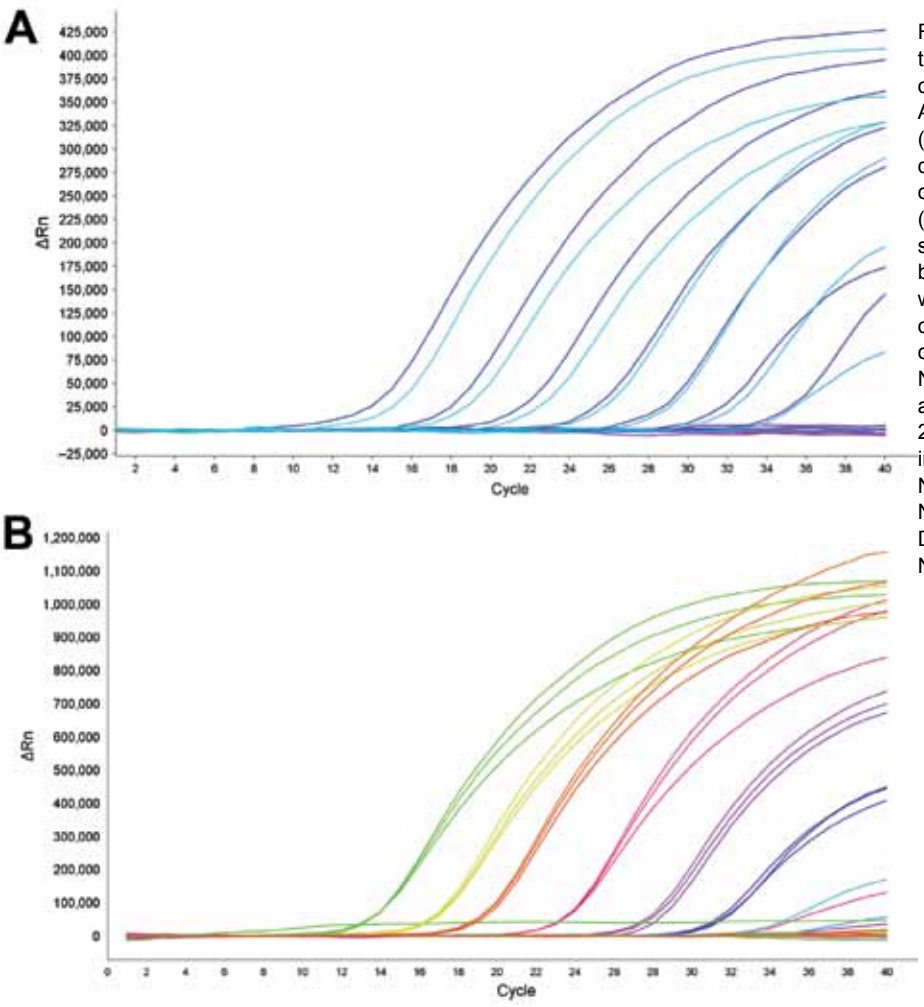


Figure. Dynamic range of reverse transcription PCR for detection of oseltamivir resistance in influenza A(H7N9) virus. Amplification curves (ΔRn vs. cycle number) for serial dilutions of plasmid with 292K (mutant) or R292 (wild-type) neuraminidase (NA) fragments. ΔRN is change in signal magnitude (reporter signal minus baseline signal). Assay dynamic range was linear at template concentrations of 10^2–10^8 copies/reaction. A) Detection of NA 292K mutant strain with probe N9-K: slope = -3.388, R2 = 0.997. Light and dark blue curves indicate probe NA 292K in duplicate wells. Violet curves indicate control wells. B) Detection of NA R292 wild-type strain with probe N9-R: slope = –3.672, R2 = 0.992. Different colored curves indicate probe N9-R in triplicate wells.

5 samples positive in the 292K assay and 2 samples positive in both assays. Four of 11 samples were negative in both assays. All 292K-positive samples were further confirmed as positive by Sanger sequencing of NA genes.

The 7 samples that contained the 292K mutant were obtained from 4 patients: 2 patients who died (patients 2 and 3) and 2 patients who recovered (patients 8 and 10). In our previous study, sequencing of the NA gene was not successful for the first throat swab specimens from patients 8 and 10 because of low viral load (*3*). However, in this study, the 292K mutant was found in sputum specimens from these 2 patients on days 7 or 15, respectively, after initiation of antiviral treatment. This finding suggested that influenza A(H7N9) virus mutated under the pressure of antiviral treatment, which led to failure of the virus to clear the lower respiratory tracts. These 2 patients, who were infected with the drug-resistant mutant virus, recovered from their diseases, which suggested that host immune response might play a major role in controlling the mutant virus.

Conclusions

Higher viral load in sputum samples indicated that there might be factors, including hemagglutinin (HA) binding preference, which favor greater replication in the lower respiratory tract. A similar phenomenon was observed in patients infected with the HA D222G mutant of influenza A(H1N1)pdm09 virus; this virus showed preferential replication in the lower respiratory tract and this infection was correlated with severe outcomes or deaths (*7*).

We have developed an SNP real-time RT-PCR for detection of a drug-resistant NA R292K mutant of influenza A(H7N9) virus. The sensitivity of the assay is lower than that of an HA7-specific real-time RT-PCR (i.e., 4 samples positive for influenza A(H7N9) virus were not detected by this RT-PCR). However, as a screening tool, this assay is sensitive, specific, fast, and inexpensive.

This assay had a detection threshold of 10% for a mutant strain in a mixed viral population, which is more sensitive than Sanger sequencing (detection threshold of 25% for a minor component in a mixed viral population) (*6*).

Table Detection of wild-type neuraminidase R292 and mutant 292K influenza A(H7N9) virus in clinical samples from 9 patients in China, by reverse transcription PCR*

Patient no.†	Outcome	Sample type	Time after oseltamivir treatment started, d	Time after oseltamivir treatment ended, d	292K:R292 ratio	Viral load, copies/mL‡
2	Died	NPS	9	−1	100:0	1.78×10^5
		S	9	−1	100:0	8.76×10^4
		NPS	10	−2	100:0	6.14×10^4
		S	10	−2	25.7:74.3	1.29×10^4
3	Died	NPS	13	3	–	ND
		S	13	3	–	3.03×10^3
		NPS	16	6	–	ND
		S	16	6	100:0	1.21×10^4
5	Died	NPS	7	−4	–	9.96×10^2
		S	11	0	–	ND
6	Recovered	NPS	16	−3	–	ND
		S	19	0	–	ND
7	Recovered	NPS	13	0	–	ND
		S	13	0	–	ND
8	Recovered	NPS	7	0	–	ND
		S	7	0	100:0	1.76×10^4
10	Recovered	NPS	11	−4	–	ND
		S	15	0	91.7:8.3	5.25×10^4
15	Died	NPS	20	4	–	ND
		S	20	4	–	5.62×10^3
17	Recovered	NPS	11	0	–	ND
		S	11	0	–	3.06×10^4

*NPS, nasopharyngeal swab specimen; S, sputum specimen; –, no ratio because samples had negative results; ND, not detected.
†Patient identification was identical with that used by Hu et al. (*3*). Patients 15 and 17 are initially reported in the current study.
‡Method used to determine viral load was reported by Hu et al. (*3*).

This assay will help clinicians monitor emergence of drug-resistant virus strains during treatment of patients with NA inhibitors to prevent persistent viral replication and severe inflammatory reactions.

This study was supported by the National Megaprojects of China for Infectious Diseases (2012ZX10004211 and 2014ZX10004002-005), the Ministry of Science and Technology (KJYJ-2013-01-01), and the Shanghai Municipal Health and Family Planning Commission (2013QLG002).

Dr Wang is an associate professor in the Pathogen Diagnosis and Biosafety Department, Shanghai Public Health Clinical Center affiliated with Fudan University, Shanghai, China. Her primary research interest is genomic features of respiratory pathogens.

References

1. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 2013;368:1888–97. http://dx.doi.org/10.1056/NEJMoa1304459
2. Gao HN, Lu HZ, Cao B, Du B, Shang H, Gan JH, et al. Clinical findings in 111 cases of influenza A (H7N9) virus infection. N Engl J Med. 2013;368:2277–85. http://dx.doi.org/10.1056/NEJMoa1305584
3. Hu Y, Lu S, Song Z, Wang W, Hao P, Li J, et al. Association between adverse clinical outcome in human disease caused by novel influenza A H7N9 virus and sustained viral shedding and emergence of antiviral resistance. Lancet. 2013;381:2273–9. http://dx.doi.org/10.1016/S0140-6736(13)61125-3
4. Sleeman K, Guo Z, Barnes J, Shaw M, Stevens J, Gubareva LV. R292K substitution and drug susceptibility of influenza A(H7N9) viruses. Emerg Infect Dis. 2013;19:1521–4. http://dx.doi.org/10.3201/eid1909.130724
5. Watanabe T, Kiso M, Fukuyama S, Nakajima N, Imai M, Yamada S, et al. Characterization of H7N9 influenza A viruses isolated from humans. Nature. 2013;501:551–5. http://dx.doi.org/10.1038/nature12392
6. Liu CM, Driebe EM, Schupp J, Kelley E, Nguyen JT, McSharry JJ, et al. Rapid quantification of single-nucleotide mutations in mixed influenza A viral populations using allele-specific mixture analysis. J Virol Methods. 2010;163:109–15. http://dx.doi.org/10.1016/j.jviromet.2009.09.007
7. Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W, et al. Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. J Virol. 2010;84:12069–74. http://dx.doi.org/10.1128/JVI.01639-10

Address for correspondence: Yunwen Hu, Pathogen Diagnosis and Biosafety Department, Shanghai Public Health Clinical Center, Fudan University, No. 2901 Caolang Rd, Jinshan District, Shanghai, China; email: ywhu0117@126.com

Novel Avian Influenza A(H7N9) Virus in Tree Sparrow, Shanghai, China, 2013

Baihui Zhao,[1] Xi Zhang,[1] Wenfei Zhu,[1] Zheng Teng, Xuelian Yu, Ye Gao, Di Wu, Enle Pei, Zhengan Yuan, Lei Yang, Dayan Wang, Yuelong Shu, and Fan Wu

In spring 2013, influenza A(H7N9) virus was isolated from an apparently healthy tree sparrow in Chongming Dongping National Forest Park, Shanghai City, China. The entire gene constellation of the virus is similar to that of isolates from humans, highlighting the need to monitor influenza A(H7N9) viruses in different species.

Since its emergence in China in February 2013, avian influenza A(H7N9) virus has resulted in 217 human infections and 57 deaths (*1*). The biological features of the virus and its pandemic potential have caused global concern (*2*). Although the epidemic declined quickly after the closure of live poultry markets in China in April 2013, new cases in humans have reemerged since October 2013. The number of new cases has increased sharply since January 1, 2014, paralleling the peak of the first wave (*1*,*3*,*4*), indicating that subtype H7N9 viruses were circulating asymptomatically among natural hosts. Sequence data indicated that the hemagglutinin gene of this novel subtype H7N9 virus might originate from a subtype H7N3 virus in ducks and that the neuraminidase gene probably originated from a subtype H7N9 virus in wild birds (*5*) or ducks or chickens (*6*,*7*). These data suggest that wild birds might play a role in the emergence of subtype H7N9 viruses, similar to the role they played in the geographic spread of avian subtype H5N1 viruses (*8*). However, although avian influenza A(H7N9) viruses have been isolated from chickens and pigeons, to our knowledge, none have been isolated from wild birds. To better understand the role of wild birds in the emergence and potential dissemination of subtype H7N9 viruses, during spring of 2013, the Shanghai Municipal Center for Disease Control and Prevention, in collaboration with the Shanghai Wildlife Conservation and Management Center, investigated influenza A(H7N9) virus infection among wild birds in Shanghai.

The Study

During April 10–May 15, a total of 2,198 fecal, tissue, cloacal swab, and tracheal swab samples were collected from wild birds in Shanghai. Trained staff captured healthy birds with an approved trapping method, collected samples, and released the birds. Tissue samples were collected from naturally dead wild birds. Information on bird species and sampling places are listed in online Technical Appendix Tables 1 and 2 (wwwnc.cdc.gov/EID/article/20/5/13-1707-Techapp1.pdf). RNA was extracted from each sample and tested by using influenza A universal real-time PCR according to the standard operating procedure of the World Health Organization (*9*). Influenza A virus–positive specimens were further subtyped by reverse transcription PCR with an avian influenza A virus subtype primer set reported previously (*10*). Of the 2,198 samples, 28 were positive for influenza A virus. One tracheal sample from an apparently healthy tree sparrow was positive for the novel subtype H7N9 virus, whereas the cloacal swab samples from this bird were negative. The positive sample was inoculated into 11-day-old specific pathogen free embryonated chicken eggs for virus isolation. The isolated virus was termed A/tree sparrow/Shanghai/01/2013 (H7N9). The tree sparrow had been collected from a forest on Chongming Dongping Forest Park, which is 47 km from Dongtan National Nature Reserve, a winter habitat for wild migratory birds (Figure 1).

To explore the genetic relationships between this sparrow-derived influenza A(H7N9) virus and other viruses from humans and poultry, we amplified total genomic segments by using viral RNA directly isolated from the original specimen with the primer sets listed in online Technical Appendix Table 3 and sequenced by Sunny Biotech Co., Ltd. (Shanghai, China). The Chinese National Influenza Center performed the sequencing by using RNA from chicken embryonated cultured viruses in an ABI 3730xl automatic DNA analyzer (Life Technologies, Foster City, CA, USA). Full-genome sequences from the original sample and the embryonated chicken egg isolation were deposited in GenBank (accession nos. KF609524–KF609531 and KJ508887–KJ508894). To facilitate the phylogenetic analysis, we downloaded sequences of the novel subtype H7N9 viruses from 2013 and the avian subtype H7N9 viruses from before 2013 from the Global Initiative on Sharing Avian Influenza Data (http://platform.gisaid.org/

Author affiliations: Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China (B. Zhao, X. Zhang, Z. Teng, X. Yu, Y. Gao, Z. Yuan, F. Wu); National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China (W. Zhu, L. Yang, D. Wang, Y. Shu); and Shanghai Wildlife Conservation and Management Center, Shanghai (D. Wu, E. Pei)

DOI: http://dx.doi.org/10.3201/eid2005.131707

[1]These authors contributed equally to this article.

Figure 1. Location of tree sparrow from which novel avian influenza A(H7N9) virus was isolated: Chongming National Dongping Forest Park of Shanghai (yellow solid circle), which is located in the Australia–East Asia migratory wild bird flyway. Top right: sampling locations in Shanghai City. Bottom right: sampling location of influenza A(H7N9)–positive tree sparrow. CM, Chongming District; BS, Baoshan District; JD, Jiading District; SH center, Changning, Putuo, and Xuhui Districts; QP, Qingpu District; MH, Minhang District; SJ, Songjiang District; PD, Pudong District; FX, Fengxian District; JS, Jinshan District.

epi3/frontend#46b284). Sequence alignments were performed by using the MegAlign method of Lagergene 7.01 software (www.dnastar.com/t-megalign.aspx). Phylogenetic analysis was analyzed by using the neighbor-joining method in MEGA software version 5.10 (www.megasoftware.net).

Eight gene segments of the tree sparrow virus shared most (≥99.3%) similarities with subtype H7N9 virus isolates from humans. Phylogenetic analysis of hemagglutinin genes revealed that subtype H7N9 viruses could be classified into Eurasia and North America lineages. The subtype H7N9 virus in this study shared the same influenza lineage with all novel subtype H7N9 viruses from humans and poultry (Figure 2). Like the hemagglutinin genes, the other 7 genes showed the same evolutionary pattern (online Technical Appendix Figure 1). Homology and phylogenetic analyses indicated that the genetic constellation of the tree sparrow–derived subtype H7N9 virus is similar to that of novel subtype H7N9 avian influenza viruses isolated from humans and poultry in this region.

According to genetic signatures, the tree sparrow–derived subtype H7N9 virus acquired the ability to bind to human-like receptors, for which substitutions G186V and Q226L in hemagglutinin protein (H3) are responsible, similar to most human and avian subtype H7N9 viruses. A 69-73–aa deletion was also found in its neuraminidase gene (N2). Amino acid 292R was maintained in neuraminidase genes, indicating its sensitivity to neuraminidase inhibitors. However, an S31N mutation in the matrix 2 protein confers resistance to adamantine. Asp at polymerase basic 2 protein (PB2) residue 701 was associated with reduced transmissibility. Mixed E/K at residue 627 in PB2 and V/I at residue 31 in matrix 1 protein were detected from the original sample (online Technical Appendix Figure 2). Because all previously reported influenza A (H7N9) viruses isolated from birds or the environment acquired PB2 627E, the mixed amino acids of 627E/K in the sample from the tree sparrow suggested that the PB2 E627K substitution might have occurred during replication of the virus in birds.

Conclusions

The high similarity of genes from the avian influenza A(H7N9) virus from an apparently healthy tree sparrow in Shanghai and influenza A(H7N9) viruses from humans and poultry in this region indicate that avian influenza A(H7N9) virus might be transmitted from poultry to tree sparrows or vice versa. Earlier reports documented that influenza A viruses, including subtypes H5N1 and H3N2, have been isolated from sparrows (*11*,*12*). A serologic survey also suggested that rates of influenza A virus infection were high among sparrows (*13*), which might result from abundant distribution of avian influenza virus receptor SA α2,3Gal in the respiratory tracts of sparrows (*14*). The novel subtype

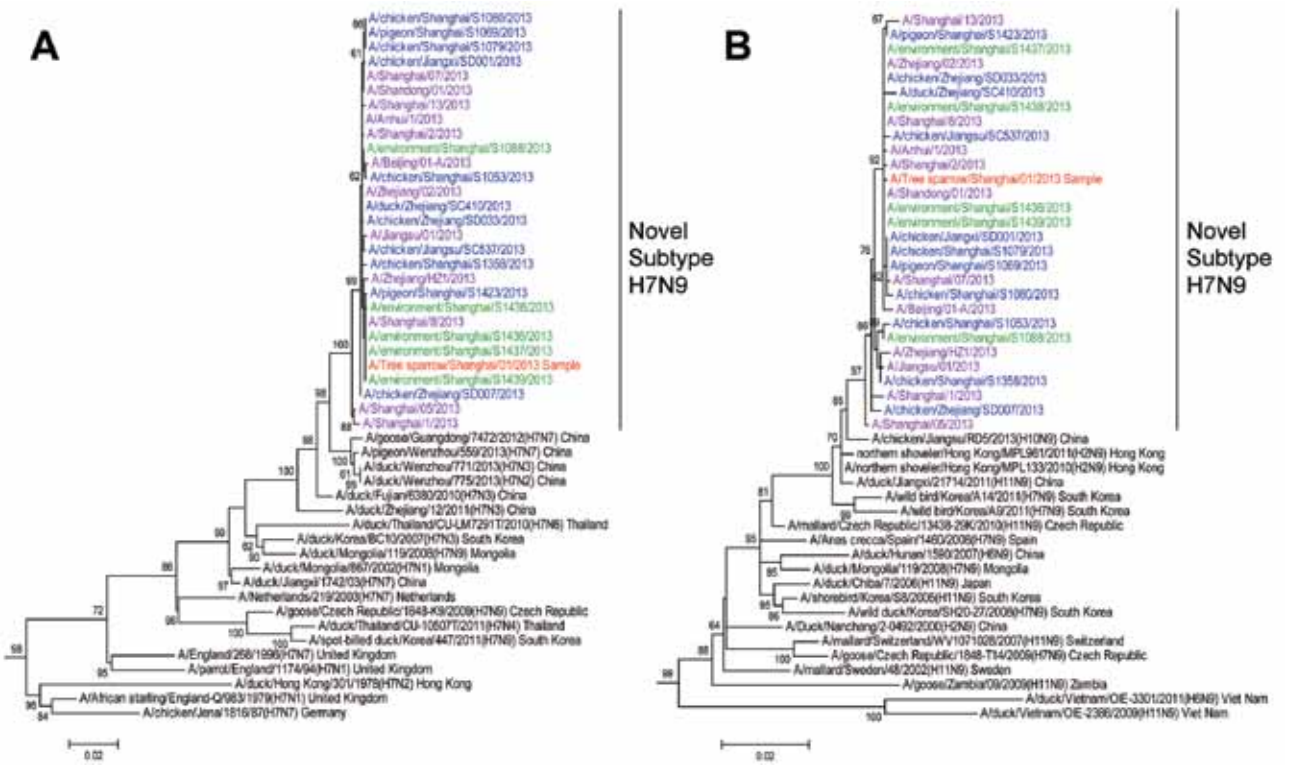


Figure 2. Phylogenetic tree of the hemagglutinin (A) and neuraminidase (B) genes of influenza A(H7N9) viruses. Multiple alignments were constructed by using the MUSCLE algorithm of MEGA software version 5.10 (www.megasoftware.net). Phylogenetic trees were constructed by using the neighbor-joining method with bootstrap analyses of 1,000 replications. Bootstrap values >60% are shown in the nodes. Sequences of human influenza A(H7N9) viruses are shown in purple, novel subtype H7N9 viruses from poultry (chickens, ducks, and pigeons) in blue, novel subtype H7N9 viruses from the environment in green, and novel subtype H7N9 viruses from wild birds in red. Scale bar indicates base substitutions per site.

H7N9 virus expands not only the number of influenza virus subtypes that infect tree sparrows but also range of hosts for subtype H7N9 viruses. Our finding of only 1 subtype H7N9–positive sample among 2,198 samples is consistent with recent findings that subtype H7N9 in wild birds is rare (*15*).

Tree sparrows are abundant and widely distributed in China. They are frequently in contact with humans and poultry. Prevalence of avian influenza viruses among tree sparrows could increase opportunities for them to carry influenza viruses from aquatic birds to domestic farms and even to humans. Hence, such expansion of influenza A(H7N9) virus host ranges undoubtedly increased the seriousness of the threat of this novel subtype.

Tree sparrows have been shown to be susceptible to influenza A(H5N1) viruses, and they might have the ability to disseminate subtype H5N1 viruses (*11*). Dongping National Forest Park, where the novel subtype H7N9–positive tree sparrow was captured, is on Chongming Island, China's third largest island, which is located in the Australia–East Asia migratory wild bird flyway. Dongping National Forest Park is adjacent to Dongtan National Nature Reserve, where hundreds of species of migratory and domestic birds gather for winter. Whether migratory birds became infected through contact with tree sparrows and then disseminated subtype H7N9 virus to other geographic regions merits further investigation. Isolation of novel influenza A(H7N9) virus in a tree sparrow emphasizes the need to expand influenza surveillance to not only domestic birds but also wild and terrestrial birds.

Acknowledgments

We thank the data submitters from the Global Initiative on Sharing Avian Influenza Data for their H7N9 sequences and Ren Lili for her assistance with the sequencing.

This study was supported by Shanghai Municipal Commission of Health and Family Planning (nos. 2013QLG001 and 2013QLG003), Ministry of Science and Technology of People's Republic of China (no. KJYJ-2013-01-01), and Science and Technology Committee of Shanghai Municipality (no. 10XD1403600).

Dr Zhao works in the microbiology laboratory of the Shanghai Municipal Center for Disease Control and Prevention. Her research interests include respiratory viruses and emerging diseases.

References

1. World Health Organization. Global alert and response. Avian influenza A(H7N9) virus [cited 2014 Jan 20]. http://www.who.int/csr/don/archive/year/2014/en/index.html
2. Zhou J, Wang D, Gao R, Zhao B, Song J, Qi X, et al. Biological features of novel avian influenza A (H7N9) virus. Nature. 2013;499:500–3. http://dx.doi.org/10.1038/nature12379
3. World Health Organization. Avian influenza A (H7N9) in China [cited 2014 Jan 22]. http://www.wpro.who.int/china/en/index.html
4. Center for Infectious Disease Research and Policy. H7N9 cases in China surge past 200 [cited 2014 Jan 21]. http://www.cidrap.umn.edu/news-perspective/2014/01/h7n9-cases-china-surge-past-200
5. Liu D, Shi W, Shi Y, Wang D, Xiao H, Li W, et al. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet. 2013;381:1926–32. http://dx.doi.org/10.1016/S0140-6736(13)60938-1
6. Lam TT, Wang J, Shen Y, Zhou B, Duan L, Cheung CL, et al. The genesis and source of the H7N9 influenza viruses causing human infections in China. Nature. 2013;502:241–4. http://dx.doi.org/10.1038/nature12515
7. Lee RT, Gunalan V, Van TD, Le LT, Eisenhaber F, Maurer-Stroh S. A new piece in the puzzle of the novel avian-origin influenza A (H7N9) virus. Biol Direct. 2013;8:26. http://dx.doi.org/10.1186/1745-6150-8-26
8. Fang LQ, de Vlas SJ, Liang S, Looman CW, Gong P, Xu B, et al. Environmental factors contributing to the spread of H5N1 avian influenza in mainland China. PLoS ONE. 2008;3:e2268. http://dx.doi.org/10.1371/journal.pone.0002268
9. World Health Organization. Manual for the laboratory diagnosis and virological surveillance of influenza [cited 2014 Jan 22]. http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/ index.html
10. Tsukamoto K, Javier PC, Shishido M, Noguchi D, Pearce J, Kang HM, et al. SYBR green-based real-time reverse transcription-PCR for typing and subtyping of all hemagglutinin and neuraminidase genes of avian influenza viruses and comparison to standard serological subtyping tests. J Clin Microbiol. 2012;50:37–45. http://dx.doi.org/10.1128/JCM.01195-11
11. Liu Q, Ma J, Kou Z, Pu J, Lei F, Li T, et al. Characterization of a highly pathogenic avian influenza H5N1 clade 2.3.4 virus isolated from a tree sparrow. Virus Res. 2010;147:25–9. http://dx.doi.org/10.1016/j.virusres.2009.09.014
12. Han Y, Hou G, Jiang W, Han C, Liu S, Chen J, et al. A survey of avian influenza in tree sparrows in China in 2011. PLoS ONE. 2012;7:e33092. http://dx.doi.org/10.1371/journal.pone.0033092
13. Arenas A, Carranza J, Perea A, Miranda A, Maldonado A, Hermoso M. Type A influenza viruses in birds in southern Spain: serological survey by enzyme-linked immunosorbent assay and haemagglutination inhibition tests. Avian Pathol. 1990;19:539–46. http://dx.doi.org/10.1080/03079459008418706
14. Gutiérrez RA, Sorn S, Nicholls JM, Buchy P. Eurasian tree sparrows, risk for H5N1 virus spread and human contamination through Buddhist ritual: an experimental approach. PLoS ONE. 2011;6:e28609. http://dx.doi.org/10.1371/journal.pone.0028609
15. Olson SH, Gilbert M, Cheng MC, Mazet JA, Joly DO. Historical prevalence and distribution of avian influenza virus A(H7N9) among wild birds. Emerg Infect Dis. 2013;19:2031–3. http://dx.doi.org/10.3201/eid1912.130649

Address for correspondence: Fan Wu, Shanghai Municipal Center for Disease Control and Prevention, No. 1380 West Zhongshan Rd, Changning District, Shanghai 200336, China; email: fwu@scdc.sh.cn

Shigella spp. with Reduced Azithromycin Susceptibility, Quebec, Canada, 2012–2013

Christiane Gaudreau, Sapha Barkati, Jean-Michel Leduc, Pierre A. Pilon, Julie Favreau, and Sadjia Bekal

During 2012–2013 in Montreal, Canada, 4 locally acquired *Shigella* spp. pulse types with the *mph*(A) gene and reduced susceptibility to azithromycin were identified from 9 men who have sex with men, 7 of whom were HIV infected. Counseling about prevention of enteric sexually transmitted infections might help slow transmission of these organisms.

Shigella spp. are transmitted directly from person to person or indirectly by low-inoculum infection (*1*). Among men who have sex with men (MSM), *Shigella* spp. are mostly transmitted sexually; clusters of such cases have been documented in Montreal and surrounding neighborhoods (*2*,*3*). Azithromycin is an alternative treatment for multidrug-resistant *Shigella* spp. infections in adults and children, but routine testing for azithromycin susceptibility is not yet standardized and recommended (*1*,*4*–*6*). In the United States, azithromycin MICs for 392 wild-type *Shigella* strains isolated in 2005–2006 were estimated to be 4–16 mg/L; the azithromycin MIC for 90% of the isolates was 8 mg/L (*7*).

The Study

In December 2012, the microbiology laboratory of the Centre Hospitalier de l'Université de Montréal–Hôpital Saint-Luc identified *Shigella* spp. with reduced susceptibility to azithromycin from 2 patients who had received this agent as treatment for shigellosis. The Montréal Public Health Department and Laboratoire de Santé Publique du Québec (LSPQ) were alerted. Retrospective and prospective laboratory surveillance was initiated to cover the period January 2011–April 2013. Laboratories routinely report shigellosis to the Montreal Public Health Department (Quebec, Canada).

Phenotypic identification of all *Shigella* spp. at the genus and species levels (*8*) was confirmed at LSPQ as described (*9*), after which serologic identification by slide agglutination (Denka Seiken Co., Ltd, Coventry, UK) was performed. Pulsed-field gel electrophoresis (PFGE) was performed at LSPQ according to international standards set by the US Centers for Disease Control and Prevention (*10*). Pulse types were determined by *Shigella* species, serotypes, and PFGE patterns. All *Shigella* spp. isolated during 2011–2013 underwent susceptibility testing for ampicillin, trimethoprim/sulfamethoxazole, and ceftriaxone by use of Vitek 2 (bioMérieux, Marcy l'Étoile, France) and for azithromycin and ciprofloxacin by use of Etest (AB Biodisk, Solna, Sweden). *Shigella* spp. with elevated MICs for azithromycin were also tested by disk diffusion for 30 μg nalidixic acid and by Etest for tetracycline and chloramphenicol. Vitek 2 and Etest susceptibility testing was performed as recommended by the manufacturers, and quality control strains gave expected results. The *mph*(A) gene, which codes for the macrolide 2′-phosphotransferase, was detected by PCR, as described (*11*).

After receiving ethics approval from the Centre Hospitalier de l'Université de Montréal–Hôpital Saint-Luc, we reviewed hospital charts and public health investigation files of patients who were harboring *Shigella* spp. with decreased susceptibility to azithromycin. Differences were analyzed by using the Fisher exact 2-tailed test with Epi Info software, version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Statistical significance was set at $p<0.05$.

From January 1, 2011, through April 30, 2013, a total of 45 patients were infected by 46 *Shigella* spp. strains isolated from fecal samples, including 2 also isolated from blood. A total of 33 *Shigella* spp. isolates were acquired locally by 33 men, and 13 *Shigella* spp. isolates

Author affiliations: Centre Hospitalier de l'Université de Montréal–Hôpital Saint-Luc, Montreal, Quebec, Canada (C. Gaudreau, S. Barkati, J.-M. Leduc, J. Favreau); Université de Montréal, Montreal (C. Gaudreau, S. Barkati, J.-M. Leduc, P.A. Pilon, S. Bekal); Agence de la Santé et des Services Sociaux de Montréal-Santé Publique, Montreal (P.A. Pilon); and Laboratoire de Santé Publique du Québec/Institut National de Santé Publique du Québec, Sainte-Anne-de-Bellevue, Quebec, Canada (C. Gaudreau, S. Bekal)

DOI: http://dx.doi.org/10.3201/eid2005.130966

Table 1. Azithromycin susceptibility of 26 *Shigella* spp. isolates from 25 patients, Centre Hospitalier de l'Université de Montréal–Hôpital Saint-Luc, Montreal, Quebec, Canada, January 2012–April 2013*

Azithromycin susceptibility	No. infections acquired locally	No. infections acquired abroad
Reduced	10	0
Susceptible	7	9

*p = 0.0039. All patients with locally-acquired *Shigella* and 3 patients with *Shigella* infections acquired abroad were men. One patient was infected successively with 2 *Shigella* species with reduced azithromycin susceptibility. For all patients infected with *Shigella* spp. with decreased azithromycin susceptibility, the strains were isolated from feces and none from blood.

Table 2. Characteristics and antimicrobial susceptibility of 4 *Shigella* isolates with reduced azithromycin susceptibility, Montreal, Quebec, Canada, January 2012–April 2013*

Shigella species	ST	PV†	AZM, mg/L‡	AMP, mg/L	TMP/SMX, mg/L	CIP mg/L	CRO, mg/L	TET, mg/L	CHL, mg/L	NAL mm
S. flexneri	2a	15	256	≤2 (S)	≥320 (R)	0.016	≤1	≥128	0.5 (S)	27
S. flexneri	2a	16	64	≥32 (R)	≥320 (R)	0.016	≤1	≥128	128 (R)	27
S. flexneri	3a	6	>256	≥32 (R)	≤20 (S)	0.016	≤1	≥128	>256 (R)	24–28
S. sonnei	–	101, 105§	>256	≥32 (R)	≥320 (R)	0.016	≤1	≥128	>256 (R)	23–27

*The susceptibility and resistance break points for AMP, CIP, TMP/SMX, CRO, TET, CHL, and NAL were Clinical and Laboratory Standards Institute *Enterobacteriaceae* break points (*12*). ST, serotype; PV, pulsovar; AZM, azithromycin; AMP, ampicillin; TMP/SMX, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; CRO, ceftriaxone; TET, tetracycline; CHL, chloramphenicol; NAL, nalidixic acid; S, susceptible; R: resistant; –, not applicable.
†PV was determined by *Xba*I and *Bln*I pulsed-field gel electrophoresis patterns.
‡The criterion for elevated azithro MIC was >16 mg/L (*13*).
§*S. sonnei* PVs 101 and 105 were related and had 2 different pulsed-field gel electrophoresis bands.

were acquired abroad, outside Canada, in the week before symptom onset, by 6 men and 7 women (p = 0.00003).

From January 2012 through April 2013, infection with 4 *Shigella* spp. pulse types with decreased azithromycin susceptibility was locally acquired by 9 patients (mean age 45 years, range 29–55 years) (Tables 1, 2). Among these patients, 1 HIV-positive man was infected successively with 2 *Shigella* species with reduced azithromycin susceptibility, 11 months apart, resulting in a total of 10 infections (Figure). All 9 men reported having had sex with men, and 7 were HIV positive. CD4 cell counts were 320×10^6 cells/L for 1 HIV-positive patient and 420–540 ´ 10^6 cells/L for the other 6. HIV viral load was <40 copies/mL for 3 of the 6 patients for whom data were available and 58–90,074 copies/mL for the other 3. During the previous 6 years, 7 men for whom these data were available had experienced 1–7 (median 4) other sexually transmitted diseases. Of the 9 men, 4 reported use of sex venues and none had worked in daycare centers or as a food handler. All 9 patients received follow-up care at medical clinics outside the hospital, but 4 patients received care at the emergency room for 24–48 hours. For treatment, 4 patients received ciprofloxacin and 2 received azithromycin; antimicrobial drug treatment is unknown for the other 3 patients. For these 9 men, information was unknown with regard to receipt of azithromycin before illness onset, clinical outcome data, and antimicrobial drug treatment failure. Among the *Shigella* pulse types with reduced susceptibility to azithromycin, 2 originated from outbreaks among MSM (Figure), which are being investigated by Quebec public health departments and LSPQ.

During the 2011–2013 surveillance period, azithromycin MICs for 35 of 36 *Shigella* spp. isolates with no reduced azithromycin susceptibility were 2–8 mg/L, and the MIC for 1 isolate was 16 mg/L; this latter isolate was negative by PCR for *mph*(A), and the other 35 isolates were not tested. The 10 *Shigella* spp. isolates with reduced azithromycin susceptibility had azithromycin MICs ≥64 mg/L and were positive for the *mph*(A) gene by PCR. The 3 *S. flexneri* and 1 *S. sonnei* pulse types were susceptible to nalidixic acid, ciprofloxacin, and ceftriaxone (Table 2); 3 pulse types were resistant to ampicillin, trimethoprim/sulfamethoxazole, or chloramphenicol; and 4 pulse types were resistant to tetracycline (Table 2). During 2012–2013, *Shigella* spp. with reduced azithromycin susceptibility represented 57.1% of 7 locally acquired pulse types (data not shown). Pulse-Net Canada *Xba*I and *Bln*I pattern designations were SFXXAI.0205/SFXBNI.0092 and SFXXAI.0204/SFXBNI.0093 for *S. flexneri* serotype 2a pulsovars 15 and 16, respectively; SFXXAI.0193/SFXBNI.0084 for *S. flexneri* serotype 3a pulsovar 6; SSOXAI.0395/SS0BNI.0020 for *S. sonnei* pulsovar 101; and SSOXAI.0174/SSOBNI.0176

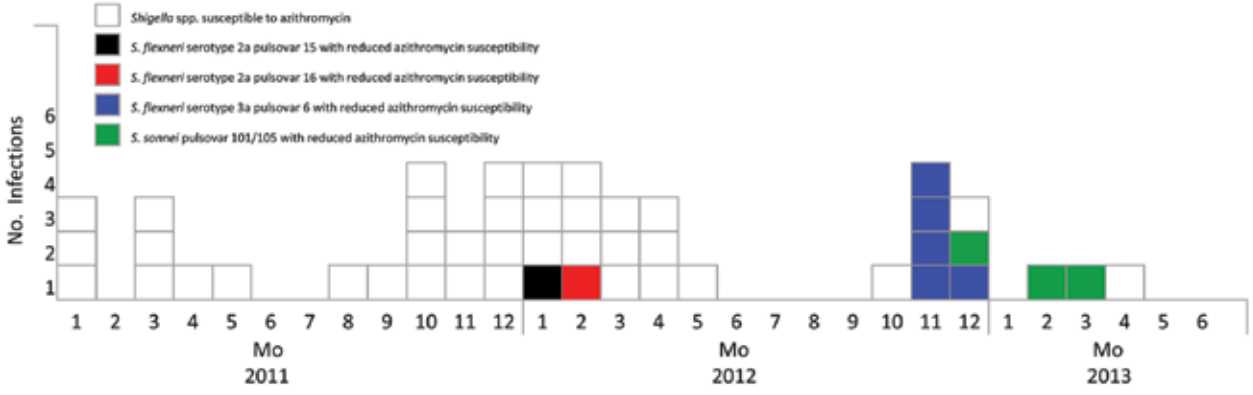


Figure. Distribution of *Shigella* spp. infections by sample date and years, Montreal, Quebec, Canada, January 2011–April 2013.

for *S. sonnei* pulsovar 105. No PFGE matches were identified in isolates from other Canada provinces.

Conclusions

During 2012–2013, at the Centre Hospitalier de l'Université de Montréal–Hôpital Saint-Luc, 10 infections with 1 of the 4 *Shigella* spp. pulse types with reduced azithromycin susceptibility were documented for 9 MSM, 7 of whom were HIV positive. These 4 locally acquired *Shigella* pulse types had increased azithromycin MICs of ≥64 mg/L and were positive by PCR for *mph*(A). This gene, which encodes macrolide-inactivating 2′-phosphotransferase, occurs on various plasmids (*7*). It has been documented in many aerobic gram-negative rods, such as *Escherichia coli* and *Shigella* spp. (*14*). This gene was harbored by all *Shigella* spp. with azithromycin MICs >16 mg/L (*7*,*13*–*15*). Azithromycin treatment failure has been reported for patients who received this drug for infection with such isolates (*14*). In our study, the acquisition of this gene by >50% of locally acquired *Shigella* spp. pulse types, infecting MSM over 15 months, is a concern in view of the potentially rapid development of reduced *Shigella* spp. susceptibility to azithromycin. For facilitation of clinical decision making and surveillance, azithromycin susceptibility break points for *Enterobacteriaceae* should be standardized (*12*). MSM should be counseled about prevention of enteric sexually transmitted infections; prevention measures include handwashing and using barriers during oral, anal, and genital sex (*2*,*3*). Such counseling might lead to behavior changes that might help slow the transmission of enteric sexually transmitted infections, including *Shigella* spp. infections with reduced azithromycin susceptibility.

Acknowledgments

We thank Cécile Tremblay, Robert Allard, and Ovid M. Da Silva for editorial work on the manuscript.

Dr Gaudreau is a clinical microbiologist and infectious diseases physician at Centre Hospitalier de l'Université de Montréal–Hôpital Saint-Luc in Montreal and a clinical titular professor at the Département de Microbiologie, Infectiologie et Immunologie de l'Université de Montréal. Her main research interests are epidemiology and antimicrobial drug susceptibility of enteric bacteria.

References

1. DuPont HL. *Shigella* species (bacillary dysentery). In: Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases, 7th ed. Philadelphia: Elsevier Churchill Livingstone; 2010. p. 2905–10.
2. Gaudreau C, Bruneau A, Ismaïl J. Outbreak of *Shigella flexneri* and *Shigella sonnei* enterocolitis in men who have sex with men, Québec, 1999 to 2001. Can Commun Dis Rep. 2005;31:85–90.
3. Gaudreau C, Ratnayake R, Pilon PA, Gagnon S, Roger M, Lévesque S. Ciprofloxacin-resistant *Shigella sonnei* among men who have sex with men, Canada, 2010. Emerg Infect Dis. 2011;17:1747–50. http://dx.doi.org/10.3201/eid1709.102034
4. American Academy of Pediatrics. *Shigella* infections. In: Pickering LK, Baker CJ, Kimberlin DW, Long SS, editors. Red book, 2012 Report of the Committee on Infectious Diseases, 29th ed. Elk Grove Village (IL): The Academy; 2012. p. 645–7.
5. World Health Organization. Guidelines for the control of shigellosis, including epidemics due to *Shigella dysenteriae* type 1. Geneva: The Organization; 2005.
6. Khan WA, Seas C, Dhar U, Salam MA, Bennish ML. Treatment of shigellosis: V. Comparison of azithromycin and ciprofloxacin. Ann Intern Med. 1997;126:697–703. http://dx.doi.org/10.7326/0003-4819-126-9-199705010-00004
7. Howie RL, Folster JP, Bowen A, Barzilay EJ, Whichard JM. Reduced azithromycin susceptibility in *Shigella sonnei*, United States. Microb Drug Resist. 2010;16:245–8. http://dx.doi.org/10.1089/mdr.2010.0028
8. Nataro JP, Bopp CA, Fields PI, Kaper JB, Strockbine NA. *Escherichia, Shigella,* and *Salmonella.* In: Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW, editors. Manual of clinical microbiology, 10th ed. Washington (DC): American Society for Microbiology; 2011. p. 603–26.
9. Ewing WH. The genus *Shigella*. In: Ewing W, Edwards PR, editors. Edwards and Ewing's identification of *Enterobacteriaceae,* 4th ed. New York: Elsevier Scientific Publishing Co., Inc.; 1986. p. 135–72.
10. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella* and *Shigella* for PulseNet. Foodborne Pathog Dis. 2006;3:59–67. http://dx.doi.org/10.1089/fpd.2006.3.59
11. Ojo KK, Ulep C, Van Kirk N, Luis H, Bernardo M, Leitao J, et al. The *mef*(A) gene predominates among seven macrolide resistance genes identified in gram-negative strains representing 13 genera, isolated from healthy Portuguese children. Antimicrob Agents Chemother. 2004;48:3451–6. http://dx.doi.org/10.1128/AAC.48.9. 3451-3456.2004
12. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 23th informational supplement; no. M100–S23, vol. 33, no. 1. Wayne (PA): The Institute; 2013.
13. Sjölund Karlsson M, Bowen A, Reporter R, Folster JP, Grass JE, Howie RL, et al. Outbreak of infections caused by *Shigella sonnei* with reduced susceptibility to azithromycin in the United States. Antimicrob Agents Chemother. 2013;57:1559–60. http://dx.doi.org/10.1128/AAC.02360-12
14. Boumghar-Bourtchai L, Mariani-Kurkdjian P, Bingen E, Filliol I, Dhalluin A, Ifrane SA, et al. Macrolide-resistant *Shigella sonnei.* Emerg Infect Dis. 2008;14:1297–9. http://dx.doi.org/10.3201/eid1408.080147
15. Centers for Disease Control and Prevention. Outbreak of infections caused by *Shigella sonnei* with decreased susceptibility to azithromycin—Los Angeles, California, 2012. MMWR Morb Mortal Wkly Rep. 2013;62:171.

Address for correspondence: Christiane Gaudreau, Microbiologie Médicale et Infectiologie, CHUM-Hôpital Saint-Luc, 1058 rue Saint-Denis, Montreal, Quebec, Canada H2X 3J4; email: christiane.gaudreau.chum@ssss.gouv.qc.ca

Influenza A(H5N2) Virus Antibodies in Humans after Contact with Infected Poultry, Taiwan, 2012

Ho-Sheng Wu, Ji-Rong Yang, Ming-Tsan Liu, Chin-Hui Yang, Ming-Chu Cheng, and Feng-Yee Chang

Six persons in Taiwan who had contact with poultry infected with influenza A(H5N2) showed seroconversion for the virus by hemagglutinin inhibition or microneutralization testing. We developed an ELISA based on nonstructural protein 1 of the virus to differentiate natural infection from cross-reactivity after vaccination; 2 persons also showed seroconversion by this test.

Since 1959, highly pathogenic avian influenza A (HPAI) subtypes H5 and H7 have caused outbreaks in poultry resulting in high mortality rates and have also caused sporadic infections in humans (*1*–*3*). Some low pathogenicity avian influenza (LPAI) viruses can mutate to become HPAI virus by acquiring basic amino acid residues in the hemagglutinin (HA) cleavage site after multiple passages in chickens (*4*). In Taiwan, poultry infected by LPAI (H5N2) virus were reported during 2003–2004 and 2008–2011 (*5*–*7*), whereas HPAI (H5N2) viruses were first isolated in 2012 and caused subsequent outbreaks in poultry. Although >20 differences have been found in amino acids in the HA protein between the 2012 HPAI (H5N2) viruses and the 2003 LPAI (H5N2) virus (A/chicken/Taiwan/1209/2003), these viruses are antigenically similar (M.-C. Cheng, unpub. data) and related to those that circulated in Mexico in 1994 but unrelated to the subtype H5N1 viruses that reemerged in 2003 and the subtype H5N1 vaccine strain (A/Vietnam/1194/2004) (*5*).

As of December 23, 2013, influenza A(H5N2) virus had not been isolated from humans, but previous studies have provided serologic evidence for subclinical infections in persons who had frequent contacts with infected animals (*3*,*8*–*11*). We therefore investigated the possibility of infection among persons who were exposed to HPAI (H5N2) virus during outbreaks in chicken farms in Taiwan during January–March 2012.

Author affiliations: Centers for Disease Control, Taipei, Taiwan (H.-S. Wu, J.-R. Yang, M.-T. Liu, C.-H. Yang, F.-Y. Chang); Taipei Medical University, Taipei (H.-S. Wu); Animal Health Research Institute, Taipei (M.-C. Cheng); and National Defence Medical Center, Taipei (F.-Y. Chang)

DOI: http://dx.doi.org/10.3201/eid2005.131393

The Study

For our study, we enrolled 141 persons who had close contact with poultry at 5 chicken farms that had influenza A(H5N2) outbreaks in chickens during January–March 2012. These contacts were 15 farm workers, 90 animal health officials, and 36 temporary employees who participated in culling of infected chickens; no symptoms of influenza-like illness occurred in these persons within 1 week after culling. All 15 poultry workers had been working at their poultry farms for >6 years, and most of the animal health officials had experience in stamping out infected poultry. However, for the 36 temporary employees, previous contact histories with infected chickens were unknown.

Throat swab specimens were collected from all contacts for virus detection within 7 days from the beginning of exposure to the virus, and paired serum samples were collected 21 days apart for serologic testing. Participants were offered an inactivated influenza A(H5N1) vaccine on a voluntary basis on the day the first serum specimens were collected, and vaccination histories within 1 year before the specimen collection date were recorded through oral questionnaires. A total of 102 (72.3%) of the 141 participants were vaccinated: 22 (15.6%) received influenza A(H5N1) only; 39 (27.7%) received seasonal influenza vaccine only; 41 (29.0%) received both vaccines; and 39 (27.7%) received neither (Table 1).

We found all swab specimens were negative for influenza viruses by real-time reverse transcription PCR. However, hemagglutination inhibition (HI) and/or microneutralization (MN) test results showed 7 persons had antibody titers ≥80 for subtype H5N2 virus; 6 of these persons showed seroconversion for the virus (Table 2). Elevated antibodies against subtype H3N2 or H5N1 viruses were detected in some of the 6 persons who showed seroconversion (Table 2), which suggests that positive results for subtype H5N2 could be the result of cross-reactive antibodies from previous influenza vaccinations or infections. All 6 persons who showed seroconversion for influenza A(H5N2) virus had received vaccinations for influenza A(H5N1) and seasonal influenza (Table 2). Further, persons who received both influenza vaccinations showed a significant ($p = 0.001$) geometric mean titer increase in HI antibody against influenza A(H5N2) virus in the second samples, whereas those who did not receive both vaccinations did not show a similar increase. This finding indicates these heterologous vaccinations may influence HI antibody titers against influenza A(H5N2) virus.

Table 1. HI antibody titers for influenza A(H5N2) virus in paired serum samples of 141 persons who had contact with infected poultry*

HI titers	Second sample							Influenza vaccination history during previous 12 mo			
	Total	<10	10	20	40	80	≥160	A(H5N1) only	Seasonal only	Both	None
First sample											
<10	13	0	6	7	0	0	0	0	4 (30.8)	7 (53.8)	2 (15.4)
10	57	2	4	48	3	0	0	16 (28.1)	5 (8.8)	22 (38.6)	14 (24.5)
20	32	0	5	11	15	1*	0	3 (9.4)	17 (53.1)	3 (9.4)	9 (28.1)
40	39	0	1	10	22	4*	2*	3 (7.7)	13 (33.3)	9 (23.1)	14 (35.9)
80	0	0	0	0	0	0	0	0	0	0	0
≥160	0	0	0	0	0	0	0	0	0	0	0
Total	141	2	16	76	40	5	2	22 (15.6)	39 (27.7)	41 (29.0)	39 (27.7)

*Values are no. persons or no. (%) persons. Serum samples with hemagglutination inhibition (HI) titer ≥80 underwent microneutralization testing. Criteria for determining seroconversion in HI antibodies with regard to influenza A(H5N2) viruses: 1) a ≥4-fold increase in antibody titers in the paired serum samples; and 2) HI titer ≥80 for the second sample.

To investigate whether the influenza A(H5N2) antibodies were elevated as a result of exposure to that virus or because of vaccination with heterologous influenza viruses, we determined antibody levels to influenza A(H5N2) nonstructural protein 1 (NS1) (*12*–*15*). The NS1 protein is not readily incorporated into virions used to make inactivated influenza vaccine, so a response to NS1 protein would indicate active influenza A(H5N2) infection. Paired serum samples were analyzed by ELISA plates coated with 2 peptides, $NS1^{36–48}$ (LRRDQKSLRGRGS, NS1-pA) and $NS1^{204–225}$ (RSSNENGGPPLTPKQKREMART, NS1-pB), synthesized on the basis of the NS1 protein sequences of influenza A(H5N2) virus (NS1-pA, A/chicken/Taiwan/1209/2003) and influenza A(H3N2) virus (NS1-pB, A/Taiwan/4055/2009), respectively. The NS1-pA of the 2012 influenza A(H5N2) outbreak strain has an S48N substitution that is not found in the 2003 strain.

For controls, we simultaneously analyzed 3 groups of paired serum samples with seroconversion (data not shown): 1) samples from 7 ferrets infected with different influenza virus strains (H1N1, n = 3; H3N2, n = 1; H5N1 [A/Vietnam/1194/2004], n = 1; and A[H1N1]pdm09, n = 2); 2) samples from 8 persons infected with influenza A(H1N1)pdm09 virus; and 3) samples from 9 persons who received vaccinations against influenza A(H5N1) virus. The resulting NS1 antibody responses were plotted (Figure). Five (71.4%) of 7 ferrets showed positive NS1 response against NS1-pA and all against NS1-pB (Figure, panel A), which indicates that influenza virus infection can cause a measurable anti-NS1 response after virus challenge. For influenza virus–infected persons, 3 (37.5%) of 8 showed responses against NS1-pA and NS1-pB (Figure, panel B), but for vaccinated persons, 1 (11.1%) of 9 showed responses against NS1-pA and none against NS1-pB (Figure, panel C). These patterns suggest that anti-NS1 response elicited by natural infection is stronger than that induced by vaccination.

For the group of 7 contacts we identified who had elevated influenza A(H5N2) antibodies, 2 (contacts 1 and 3) had positive NS1 antibody response against both peptides;

Table 2. Serologic test results and vaccination and occupational histories for persons who had high antibody titers against influenza A(H5N2) virus and contact with infected poultry, Taiwan, 2012*†

Cont. no.	Sample date	Tested antigens, by influenza subtype					NS1-ELISA titer		Date and type of influenza vaccination		Occupation
		H5N2		H5N1	$H1N1_{pdm09}$	H3N2					
		HI	MN	HI			NS1-pA	NS1-pB	A(H5N1)	Seasonal	
1	Mar 3	40	**<20**	80	<10	10	**1.68**	**1.4**	2012 Mar	NA	Poultry
	Mar 30	80	**230**	80	<10	10			3, 30		worker
2	Mar 5	**40**	**160**	40	20	80	0.89	0.86	2012 Mar	2011 Oct 8	Poultry
	Mar 28	**160**	**450**	80	20	80			6, 28		worker
3	Mar 5	40	**20**	80	20	40	**1.70**	**1.34**	2012 Mar	2012 Jan	Animal
	Mar 27	80	**80**	80	20	40			5	20	health official
4	Mar 5	40	**<20**	40	10	40	1.13	1.09	2012 Mar	2012 Jan	Animal
	Apr 2	80	**80**	40	10	40			5, Apr 2	20	health official
5	Mar 6	40	20	40	<10	320	1.21	1.02	2012 Mar	2011 Nov	Temp.
	Apr 2	80	30	40	<10	160			6, Apr 2	26	employee
6	Mar 6	**20**	**<20**	40	<10	**160**	1.17	1.08	2012 Mar	2012 Mar	Temp.
	Apr 2	**80**	**160**	80	<10	**640**			6, Apr 2	5	employee
7	Mar 5	**40**	**<20**	**40**	<10	**40**	0.98	1.05	2012 Mar	2012 Mar	Temp.
	Apr 2	**320**	**1,280**	**160**	10	**640**			5, Apr 2	5	employee

*Cont., contact; HI, hemagglutination inhibition; MN, microneutralization; NS1, nonstructural protein 1; H1N1pdm09, pandemic influenza A(H1N1); NA, not applicable (did not receive vaccination); temp., temporary.
†Boldface indicates a ≥4-fold rise in antibody titer by HI or MN tests and a positive anti-NS1 antibody response by NS1-ELISA test between the first and second samples of paired serum samples. A positive anti-NS1 antibody response by NS1-ELISA assay was defined as each paired serum sample having a test absorbance of the second sample 30% higher than that of its first sample (ratio >1.30).

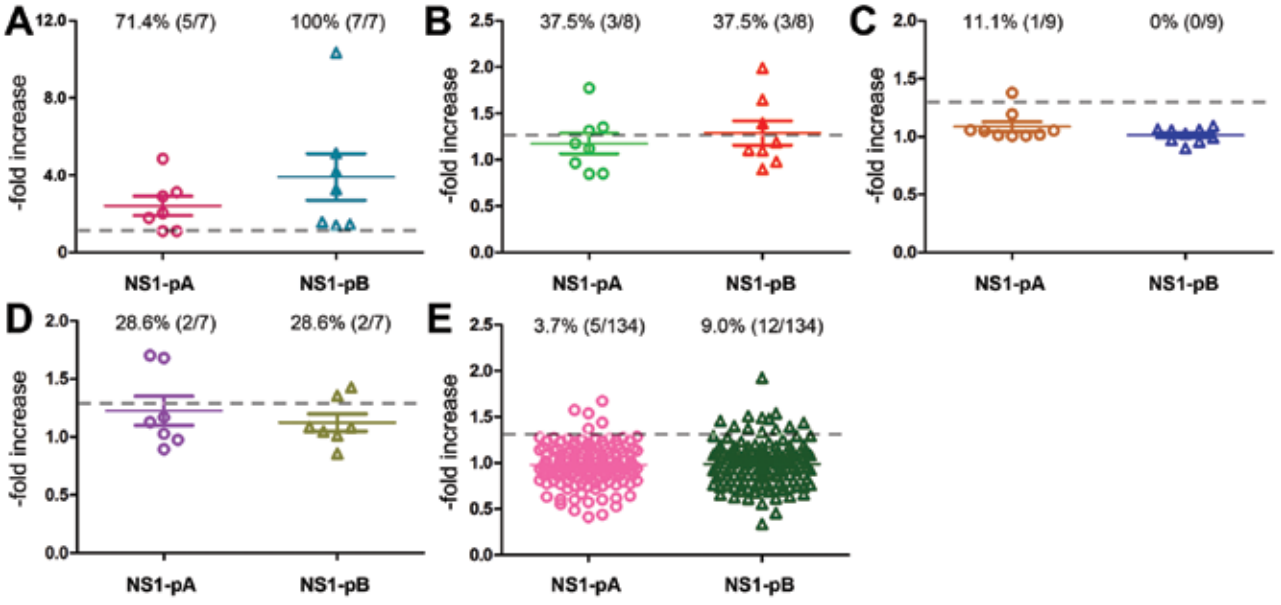


Figure. Antibody responses to 2 influenza A(H5N2) nonstructural protein 1 (NS1) peptides for paired serum samples from: A) influenza virus–infected ferrets; B) influenza virus–infected persons; C) influenza virus–vaccinated persons; D) persons in Taiwan who had contact with infected poultry during January–March 2012 and who showed seroconversion for influenza A(H5N2) virus exposure; and E) persons in Taiwan who had contact with infected poultry during January–March 2012 and who did not show seroconversion. Responses for each group were plotted by -fold increase from first to second sample against NS1-pA (circles) and NS1-pB (triangles); numbers and percentages of positive responses for each sample set are indicated above each plot. Dashed lines indicate cutoff value for defining a positive response: results for the second sample in each pair 30% higher than those for first sample.

the remaining 5 did not (Figure, panel D). These results suggest that contact 1, a poultry worker, and contact 3, an animal health official, may have experienced recent influenza infections.

To better establish the validity of using NS1 to distinguish infected from vaccinated persons, we analyzed paired serum samples for the 134 persons who did not show seroconversion for influenza A(H5N2) virus. Of these, 5 (3.7%) showed positive NS1 antibody response against NS1-pA and 12 (9.0%) against NS1-pB (Figure, panel E). This result suggests that an NS1-ELISA should not be used alone to determine influenza infection but can provide additional data to validate the results of protein-based serologic assays.

Conclusions

In this study, we sampled 141 persons exposed to poultry infected with influenza A(H5N2) virus to assess virus shedding and used multiple serologic assays (including a novel NS1 ELISA) to determine seroconversion status. We found that 6 (4.3%) persons had elevated HA antibodies detected by HI and/or MN assays; a lower percentage (1.4%, 2/141) of subclinical infections was suspected after validation by NS1 antibody assays. The NS1-peptide B was designed on the basis of influenza A(H3N2) virus; however, it also reacted with antibodies elicited by viruses of different subtypes, which suggests that consensus residues may play an essential role in forming the epitope of NS1 protein.

Our study has limitations. Patient histories of exposure to avian influenza viruses and influenza vaccination were given orally and thus may not be accurate, and mismatching between circulating viruses and antigens used in the study may have occurred. Also, recent seasonal influenza infection may interfere with the determination of subclinical infection with influenza A(H5N2) virus because the NS1 protein is remarkably conserved in type A influenza viruses.

Cross-reactive antibodies in humans elicited from heterologous influenza viruses can complicate serologic, HA-based identification of influenza subtype. The NS1-ELISA method we describe may help determine the type more readily and improve diagnosis of subclinical infection in humans. Further, our findings indicate that occupational exposure to infected poultry may pose a risk for infection in humans.

Acknowledgments

We thank Ruben Donis and Masato Tashiro for their valuable suggestions and critical appraisal of the manuscript and the many medical officers and colleagues in Regional Centers of the Taiwan CDC who participated in sample collection and onsite investigation.

This study received partial financial support from Department of Health, Taiwan (DOH101-DC-2013).

Dr Wu is director of the Taiwan National Influenza Center and of the Center for Research, Diagnostics and Vaccine Development at Taiwan CDC. His research interests are influenza viruses and enteroviruses.

References

1. Kalthoff D, Globig A, Beer M. (Highly pathogenic) avian influenza as a zoonotic agent. Vet Microbiol. 2010;140:237–45. http://dx.doi.org/10.1016/j.vetmic.2009.08.022
2. Bean WJ, Kawaoka Y, Wood JM, Pearson JE, Webster RG. Characterization of virulent and avirulent A/chicken/Pennsylvania/83 influenza A viruses: potential role of defective interfering RNAs in nature. J Virol. 1985;54:151–60.
3. Thompson PN, Sinclair M, Ganzevoort B. Risk factors for seropositivity to H5 avian influenza virus in ostrich farms in the Western Cape Province, South Africa. Prev Vet Med. 2008;86:139–52. http://dx.doi.org/10.1016/j.prevetmed.2008.03.011
4. Soda K, Cheng MC, Yoshida H, Endo M, Lee SH, Okamatsu M, et al. A low pathogenic H5N2 influenza virus isolated in Taiwan acquired high pathogenicity by consecutive passages in chickens. J Vet Med Sci. 2011;73:767–72. http://dx.doi.org/10.1292/jvms.10-0532
5. Cheng MC, Soda K, Lee MS, Lee SH, Sakoda Y, Kida H, et al. Isolation and characterization of potentially pathogenic H5N2 influenza virus from a chicken in Taiwan in 2008. Avian Dis. 2010;54:885–93. http://dx.doi.org/10.1637/9208-120609-Reg.1
6. Avian influenza (72): Taiwan LPAI H5N2. ProMed. 2011 Nov 18 [cited 2013 Sep 10]. http://www.promedmail.org, archive no. 20111122.3426.
7. Huang SY, Chen CH, Hu YJ, Shih YL, Chang CC, Chao YN, et al. Overview on prevention and control measures for human infection with HPAI H5N2 virus in Taiwan in 2012. Taiwan Epidemiol. Bullet. 2013;29:202–23.
8. Ogata T, Yamazaki Y, Okabe N, Nakamura Y, Tashiro M, Nagata N, et al. Human H5N2 avian influenza infection in Japan and the factors associated with high H5N2-neutralizing antibody titer. J Epidemiol. 2008;18:160–6. http://dx.doi.org/10.2188/jea.JE2007446
9. Yamazaki Y, Doy M, Okabe N, Yasui Y, Nakashima K, Fujieda T, et al. Serological survey of avian H5N2-subtype influenza virus infections in human populations. Arch Virol. 2009;154:421–7. http://dx.doi.org/10.1007/s00705-009-0319-7
10. Kayali G, Ortiz EJ, Chorazy ML, Gray GC. Evidence of previous avian influenza infection among US turkey workers. Zoonoses Public Health. 2010;57:265–72. http://dx.doi.org/10.1111/j.1863-2378.2009.01231.x
11. Shafir SC, Fuller T, Smith TB, Rimoin AW. A national study of individuals who handle migratory birds for evidence of avian and swine-origin influenza virus infections. J Clin Virol. 2012;54:364–7. http://dx.doi.org/10.1016/j.jcv.2012.05.001
12. Ozaki H, Sugiura T, Sugita S, Imagawa H, Kida H. Detection of antibodies to the nonstructural protein (NS1) of influenza A virus allows distinction between vaccinated and infected horses. Vet Microbiol. 2001;82:111–9. http://dx.doi.org/10.1016/S0378-1135(01)00366-2
13. Tumpey TM, Alvarez R, Swayne DE, Suarez DL. Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the nonstructural protein of influenza A virus. J Clin Microbiol. 2005;43:676–83. http://dx.doi.org/10.1128/JCM.43.2.676-683.2005
14. Avellaneda G, Mundt E, Lee CW, Jadhao S, Suarez DL. Differentiation of infected and vaccinated animals (DIVA) using the NS1 protein of avian influenza virus. Avian Dis. 2010;54(Suppl):278–86. http://dx.doi.org/10.1637/8644-020409-Reg.1
15. Takeyama N, Minari K, Kajihara M, Isoda N, Sakamoto R, Sasaki T, et al. Detection of highly pathogenic avian influenza virus infection in vaccinated chicken flocks by monitoring antibodies against non-structural protein 1 (NS1). Vet Microbiol. 2011;147:283–91. http://dx.doi.org/10.1016/j.vetmic.2010.07.002

Address for correspondence: Feng-Yee Chang, Centers for Disease Control, No. 6, Linsen S. Rd, Taipei, 10050, Taiwan; email: fychang@cdc.gov.tw

Francisella tularensis subsp. *tularensis* Group A.I, United States

Dawn N. Birdsell, Anders Johansson, Caroline Öhrman, Emily Kaufman, Claudia Molins, Talima Pearson, Miklós Gyuranecz, Amber Naumann, Amy J. Vogler, Kerstin Myrtennäs, Pär Larsson, Mats Forsman, Andreas Sjödin, John D. Gillece, James Schupp, Jeannine M. Petersen, Paul Keim, and David M. Wagner

We used whole-genome analysis and subsequent characterization of geographically diverse strains using new genetic signatures to identify distinct subgroups within *Francisella tularensis* subsp. *tularensis* group A.I: A.I.3, A.I.8, and A.I.12. These subgroups exhibit complex phylogeographic patterns within North America. The widest distribution was observed for A.I.12, which suggests an adaptive advantage.

Tularemia, caused by the bacterium *Francisella tularensis*, is a potentially severe disease that often causes unspecific symptoms; because of its low infectious dose and ease of dissemination, *F. tularensis* is considered a category A biothreat agent (*1*). Three subspecies of *F. tularensis* have been identified; *F. tularensis* subsp. *tularensis* (type A) has been identified only in North America. Numerous subtyping schemes have subdivided type A into 2 groups, A.I and A.II (*2*–*8*). Group A.II is found primarily in the western United States (*3*,*4*), whereas group A.I is found throughout the central and eastern regions of the country and sporadically in some western states (*3*,*4*,*9*).

Groups A.I and A.II differ in virulence, as do subgroups within A.I, although clinical signs and symptoms can be similar. Human infections involving A.I strains are associated with a higher fatality rate than that for infections involving A.II strains (*4*,*10*); this finding was experimentally confirmed in mice (*11*). Kugeler et al. (*10*) used pulsed-field gel electrophoresis (PFGE) to identify 2 subgroups within A.I, A1a and A1b; this study found A1b strains were associated with higher death rates and were more often isolated from human tissue types that were associated with severe disease. This difference was also experimentally confirmed in mice (*11*,*12*). However, virulence testing is not often used in clinical settings because it is slow, complicated, and expensive. Thus, molecular approaches that can rapidly assign an unknown strain to one of the recognized groups with known differences in virulence may provide valuable information to clinicians.

Because PFGE lacks the phylogenetic resolution of some other testing methods (*6*), we independently identified genetic subgroups within A.I by conducting whole-genome sequencing (WGS) of 13 A.I strains (Figure 1; Table 1, Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1559-T1.htm). The 13 strains were selected on the basis of assignment to PFGE subgroups A1a or A1b (*10*) and to maximize geographic diversity; the previously sequenced A.I strain Schu S4 (*13*) was also included. WGS data were generated, assembled, and analyzed as described in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/5/13-1559-Techapp1.pdf).

Our whole-genome phylogeny revealed 3 major subgroups within *F. tularensis* subsp. *tularensis* A.I: A.I.3, A.I.8, and A.I.12 (Figure 1). The names we assigned to these subgroups are consistent with previous phylogenetic nomenclature within *F. tularensis* (*14*). With the exception of 1 strain (ND01-1900) that was not assigned to any of the 3 subgroups, all strains previously assigned to PFGE subgroup A1a belonged to the newly designated A.I.12 subgroup (Figure 1; Table 1). In contrast, strains previously assigned to PFGE subgroup A1b were distributed among all 3 of the new subgroups (Figure 1; Table 1). We concluded that results of characterization of subgroups A1a and A1b by PFGE are not in agreement with findings of a robust whole-genome phylogeny and therefore focused the remainder of our analysis on subgroups identified by using WGS.

We observed several differences among the 3 subgroups in the whole-genome phylogeny (Figure 1). The first split separated the A.I.3 subgroup from the A.I.8 and A.I.12 subgroups; a second split separated the A.I.8 and A.I.12 subgroups. A long branch of 25 single nucleotide polymorphisms (SNPs) led to the A.I.3 subgroup, in which relatedness among the sequenced strains was moderate. A branch of 9 SNPs led to the A.I.8 subgroup, and again, relatedness among the sequenced strains was moderate. The branch leading to subgroup A.I.12 was, by comparison, much longer (37 SNPs), and the sequenced strains were separated only by 3 short branches (1–4 SNPs). This pattern of several short branches without hierarchical structuring is consistent

Author affiliations: Northern Arizona University, Flagstaff, Arizona, USA (D.N. Birdsell, E. Kaufman, T. Pearson, A. Naumann, A.J. Vogler, P. Keim, D.M. Wagner); Umeå University, Umeå, Sweden (A. Johansson); Swedish Defense Research Agency, Umeå (C. Öhrman, K. Myrtennäs, P. Larsson, M. Forsman, A. Sjödin); Centers for Disease Control and Prevention, Fort Collins, Colorado, USA (C. Molins, J.M. Petersen); Center for Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary (M. Gyuranecz); and Translational Genomics Research Institute, Flagstaff (J. Gillece, J. Schupp, P. Keim)

DOI: http://dx.doi.org/10.3201/eid2005.131559

with a recent radiation, an evolutionary process in response to adaptive change, new ecologic opportunities, or a combination of these factors.

To show more comprehensive phylogenetic patterns, we developed 16 canonical SNP (canSNP) assays as described (online Technical Appendix) and used them to screen 179 *F. tularensis* subsp. *tularensis* A.I strains selected from the collections of the Centers for Disease Control and Prevention (Fort Collins, CO, USA). We selected strains that were representative of all states where A.I infections occur and of all PFGE classification types (Table 1). One limitation of our study is that we did not analyze an equal number of strains from all regions of the country. However, our sample reflects the distribution

Table 2. Melt-MAMA primers targeting canSNPs for new phylogenetic branches in *Francisella tularensis* subsp. *tularensis* A.I in United States*

Subgroup		SchuS4†	SNP state,			Temp,
Major	Minor	position	der/anc‡	Primers, 5′ → 3′§	Con¶	°C#
NA	A.I.7	1005448**	C/T	A: TATTTCAATTTTTGCGATGGTAgGT	0.80	55
				D: ggggcggggcggggcTATTTCAATTTTTGCGATGGTAcTC	0.20	
				C: AAGTATGTTGGCAAGTAAAGTGAGAAGA	0.20	
A.I.12	NA	142781††	C/G	A: GCTTATCGCCGACATTCATCtAC	0.20	60
				D: ggggcggggcggggcgggCTTATCGCCGACATTCATCcAG	0.20	
				C: GGTATGGCAAAAAATACTTATGGTACG	0.20	
A.I.12	A.I.13	1833651‡‡	T/C	A: CTTTCAATCATGTAACCATCATTATTTAaGC	0.80	60
				D: cggggcggggcggggcggggCTTTCAATCATGTAACCATCATTATTTAgGT	0.20	
				C: CTTAATGAACTTGGTGTAATGGGTAGATA	0.20	
A.I.12	A.I.16	273622	T/C	A: AAACTTAAAAAAGAGCAAGAACTTAATGATcTC	0.60	60
				D: ggggcggggcggggcgAAACTTAAAAAAGAGCAAGAACTTAATGATaTT	0.15	
				C: CATCTTCATTAAAAGTCTTATTGTTTAAACGC	0.15	
A.I.12	A.I.15	1210286	A/G	A: TCTTAAAACATCGACACTCTCAACcTG	0.80	60
				D: ggggcggggcggggcGATCTTAAAACATCGACACTCTCAACtTA	0.20	
				C: GTATCATTCAGATCATAATGAAGCAACTATC	0.20	
A.I.12	A.I.14	1296147	T/C	A: ATCATACTGGTTATATTGGCGGTcTC	0.80	60
				D: cggggcggggcggggcggggATCATACTGGTTATATTGGCGGTgTT	0.20	
				C: GATGAGTCGCTATTAGCTTCTCGAAAG	0.20	
A.I.8	NA	1150298	G/A	A: TAGTCAATCTTGGAACTCCAGAtAA	0.75	60
				D: ggggcggggcggggcTAGTCAATCTTGGAACTCCAGAaAG	0.15	
				C: TCTATTACTCTAGGGTCAGATAGAAATTC	0.15	
A.I.8	A.I.9	1453599	C/T	A: GCTGCTGCTAGATTAGCTATgCT	0.15	60
				D: ggggcggggcggggcGCTGCTGCTAGATTAGCTATcCC	0.15	
				C: TCAAGCAATCAACAATAATTTTACTAT	0.15	
A.I.8	A.I.10	797599	T/G	A: GATCAATTGGTGGTGTTcCG	0.80	60
				D: ggggcggggcggggcGTGATCAATTGGTGGTGTTtCT	0.20	
				C: AACGTTTTATCCTCTTGAATATCAACTAT	0.20	
A.I.8	A.I.11	1278606	G/A	A: AAGGAACAAAAAACATCATCATTgCT	0.20	60
				D: ggggcggggcggggcAAAAGGAACAAAAAACATCATCATTaCC	0.20	
				C: TCATACTAACAACGGCTATTCAGGGA	0.20	
A.I.3	NA	1233898	T/G	A: GCTTGACAATATTAGCTTATAAAACTATAgTG	0.15	60
				D: ggggcggggcggggcGCTTGACAATATTAGCTTATAAAACTATAaTT	0.15	
				C: TTTTTTCCATATTTCTGTAAAAAATATACTATTATG	0.15	
A.I.3	A.I.4	830715§§	T/C	A: GTTAAGTCGGTAAGTATCGACAAaTC	0.60	60
				D: ggggcggggcggggcGTTAAGTCGGTAAGTATCGACAAgTT	0.20	
				C: CAAATCTTCTAGTATCTCTTTATCTTCAG	0.20	
A.I.3	A.I.5	113671	G/A	A: cgggcgggcgggcgggGCTTGAGTTTATTTTTTGTTTAATGTgTA	0.20	60
				D: GCTTGAGTTTATTTTTTGTTTAATGTaTG	0.20	
				C: GGACAAAACTGTGGACGTTAAGAA	0.20	
A.I.3	A.I.6	580153	G/A	A: cgggcgggcgggcgggTATAATGGTAACTCATGATCAAGAAcAA	0.20	60
				D: TTATAATGGTAACTCATGATCAAGAAaAG	0.20	
				C: ATCTGTCATGATACCAATTCTTGTCG	0.20	

*Melt-MAMA, melt–mismatch amplification mutation assay; SNP, single nucleotide polymporphism; canSNP, canonical SNP; con, concentration, μmol/L; temp, annealing temperature, °C; NA, not applicable; der, derived SNP state; anc, ancestral SNP state; D, derived allele primer; A, ancestral allele primer; C, common primer.
†Genomic position in reference A.I SchuS4 strain (GenBank accession no. NC_006570).
‡SNP states are listed according to their orientation in the SCHU S4 reference genome (GenBank accession no. AJ749949.2).
§Melt-MAMA primer sequences; primer tails and antepenultimate mismatch bases are in lower case.
¶Final concentration of each primer in Melt-MAMA genotyping assays.
#Assay annealing temperature.
**Assay designed on the reverse complement.
††SNP from (*6*).
‡‡Assay supplemented with 0.025 U of Platinum Taq DNA polymerase (Life Technologies, Invitrogen, Frederick, MD, USA).
§§SNP from (*7*).

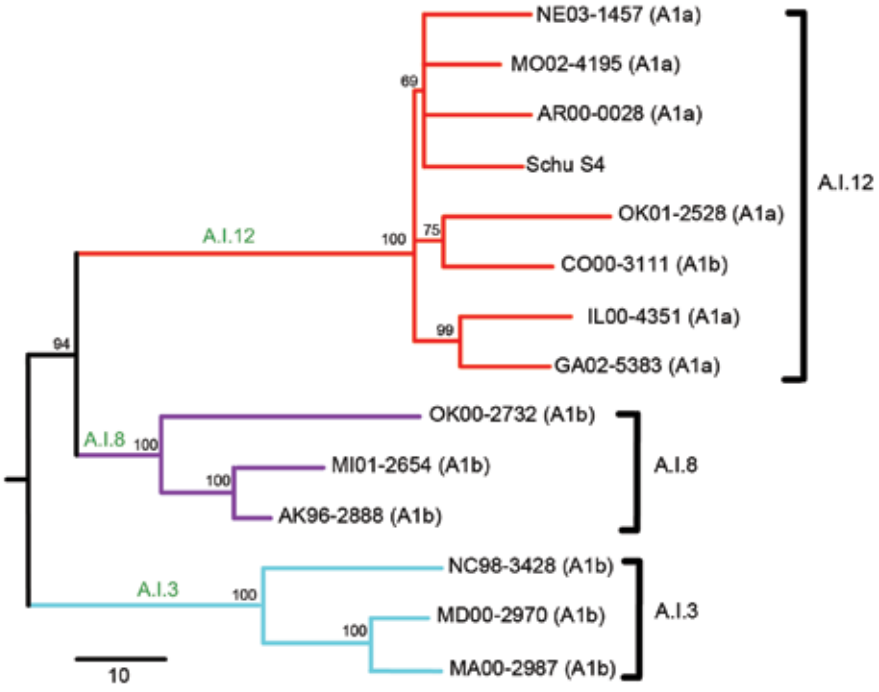


Figure 1. Neighbor-joining tree of 14 *Francisella tularensis* subsp. *tularensis* group A.I strains constructed on the basis of single-nucleotide polymorphisms (SNPs) discovered from whole-genome sequencing. Lines represent major groups within A.I: red, A.I.12; purple, A.I.8; blue, A.I.3. Branch nomenclature for each group is indicated by green text. Bootstrap values for each group and subpopulation are indicated in black font. Pulsed-field gel electrophoresis classifications (A1a and A1b) are indicated for each sequenced strain. A.I strain SchuS4 (GenBank accession no. NC_006570) was included as a reference strain. Scale bar indicates no. SNPs.

of human disease caused by *F. tularensis* subsp. *tularensis* A.I strains: prevalent in the central United States, less common in the eastern United States, and rare in the western United States (*4*). The canSNP assays were based on 12 SNP signatures (Table 2) from the whole-genome phylogeny (Figure 1) and 4 previously described SNP signatures (*6–8*). Using these assays, we assigned the 179 strains to 15 *F. tularensis* subsp. *tularensis* A.I subpopulations, including 8 intervening nodes (Figure 2, panel A). We found 6 subpopulations in the A.I.12 subgroup, 4 in A.I.8, and 4 in A.I.3 (Table 1). To identify broad phylogeographic patterns, we created maps indicating specific states where strains from the 15 subpopulations were isolated (Figure 2, panel B). Within these maps, we created boundaries corresponding to 3 regions within the United States: western, central, and eastern.

Each subgroup exhibited complex yet distinct phylogeographic patterns (Figure 2, panel B). Group A.I.12 strains, assigned to 6 subpopulations (Figure 2, panel A), were isolated throughout the United States: all 6 subpopulations were found in the central region, 3 in the western region, and 5 in the eastern region (Figure 2, panel B, top). Group A.I.8 strains, assigned to 4 subpopulations, were found in the central (3 subpopulations) and western (including Alaska and British Columbia; 3 subpopulations) regions, but only 1 strain was isolated in the eastern region (Figure 2, panel B, middle). For group A.I.3 strains, assigned to 4 subpopulations, distribution differed dramatically from the other subgroups; most strains and all 4 subpopulations occurred in the eastern region and just 1 subpopulation in the central region but none in the western region (Figure 2, panel B, bottom).

Conclusions

The occurrence of the A.I.3 subgroup in the eastern United States could be a recent or ancient event. The subgroup may have been introduced more recently from the central region to a naive niche in the eastern region through importation of rabbits (*Sylvilagus floridanus*) as recently as the 1920s (*3*); before 1937, tularemia was nearly nonexistent in the eastern region (*15*). If the introduction is recent, the current lack of A.I.3 strains in the central United States could be the result of a selective sweep that nearly eliminated this subgroup from its geographic origin. However, most strains and genetic diversity (i.e., subpopulations) within the A.I.3 subgroup are found in the eastern United States, which may reflect a more ancient history in this region involving early introduction and establishment of this subgroup east of the Appalachian Mountains, with only recent spread to the central region.

If we assume that the greatest genetic diversity in a phylogenetic context implies ancient origins, our findings suggest that the central United States is the likely geographic origin of a common ancestor to *F. tularensis* subsp. *tularensis* subgroups A.I.12 and A.I.8 and, perhaps, the A.I group as a whole. The large geographic range of the A.I.12 subgroup and the phylogenetic pattern of a long branch leading to a polytomy with genetic homogeneity point to a possible adaptive advantage for this subgroup.

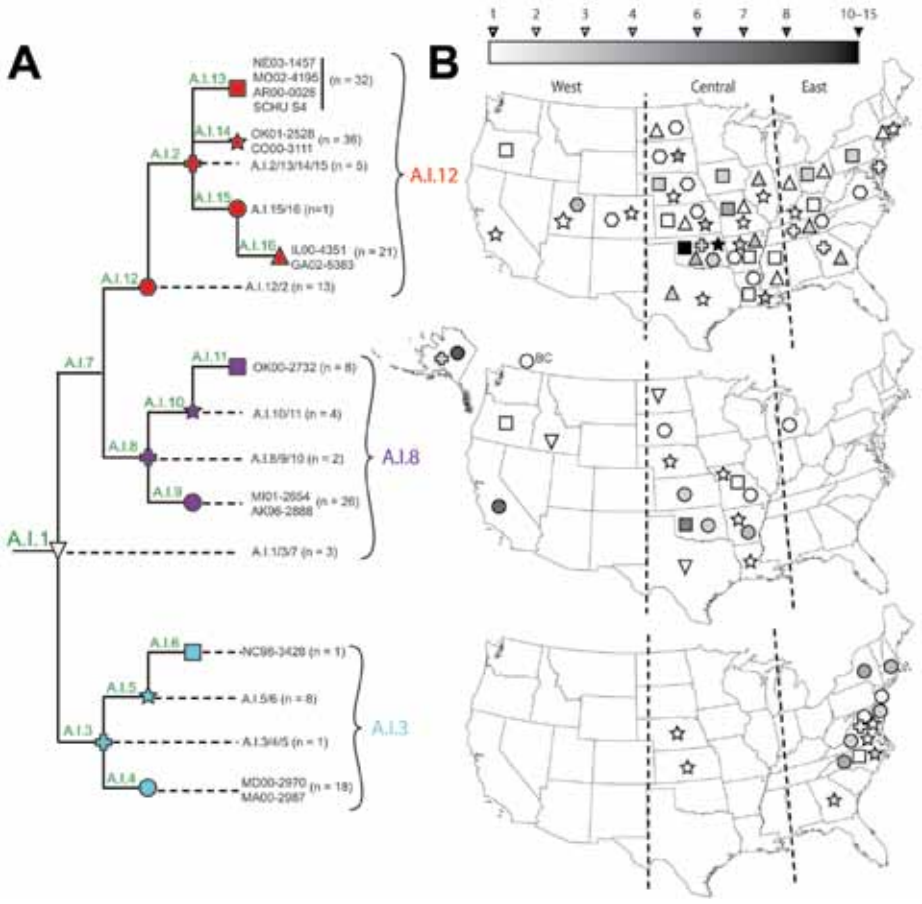


Figure 2. Geographic alignment of 179 geographically diverse *Francisella tularensis* subsp. *tularensis* A.I strains, by subgroup, United States. A) Canonical single-nucleotide polymorphism (canSNP) topology of 15 intervening and terminal subpopulations defined by screening of 16 canSNPs. Colors indicate major subgroups within A.I: red, A.I.12; purple, A.I.8; blue, A.I.3. Subpopulations are indicated by symbols; n values indicate number of strains assigned to each subpopulation. B) Geographic distribution of strains from the 15 subpopulations, shown by corresponding symbols as in panel A and aligned by subgroup (top, A.I.12; middle, A.I.8; bottom, A.I.3). Vertical lines indicate boundaries of the 3 regions: western, central, and eastern. Subgroups are mapped on the basis of geographic origin at the state level. Gradients correspond to number of strains associated with each symbol (i.e., darker symbols indicate a higher number of strains). The basal A.I.1/3/7 subgroup (inverted triangle) cannot be meaningfully assigned to 1 of the 3 main subgroups; thus, this subgroup is arbitrarily represented on the A.I.8 map. BC, British Columbia, Canada.

This advantage may be related to difference in virulence among A.I strains, as suggested by previous testing in mice of 2 A.I.12 strains that exhibited lower virulence than that of 2 A.I.3 strains (*11*). Further research is needed to determine whether the genomic differences that define this subgroup are associated with known *F. tularensis* virulence determinants.

Acknowledgments

We thank Mia Champion for her assistance with initial analyses and Laurel Respicio-Kingry for her assistance with SNP genotyping.

This work was supported in part by the US Department of Homeland Security Science and Technology Directorate through award HSHQDC-10-C-00139, the Swedish Civil Contingencies Agency through TA#014-2010-01, the Laboratory for Molecular Infection Medicine Sweden, and Västerbotten County Council. M.G. was supported by the Lendület program of the Hungarian Academy of Sciences.

Dr Birdsell is a postdoctoral fellow at the Center for Microbial Genetics and Genomics, Northern Arizona University. Her primary research interest is the evolution of *F. tularensis*.

References

1. Rotz LD, Khan AS, Lillibridge SR, Ostroff SM, Hughes JM. Public health assessment of potential biological terrorism agents. Emerg Infect Dis. 2002;8:225–30. http://dx.doi.org/10.3201/eid0802.010164
2. Johansson A, Farlow J, Larsson P, Dukerich M, Chambers E, Byström M, et al. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. J Bacteriol. 2004;186:5808–18. http://dx.doi.org/10.1128/JB.186.17.5808-5818.2004
3. Farlow J, Wagner DM, Dukerich M, Stanley M, Chu M, Kubota K, et al. *Francisella tularensis* in the United States. Emerg Infect Dis. 2005;11:1835–41. http://dx.doi.org/10.3201/eid1112.050728
4. Staples JE, Kubota KA, Chalcraft LG, Mead PS, Petersen JM. Epidemiologic and molecular analysis of human tularemia, United States, 1964–2004. Emerg Infect Dis. 2006;12:1113–8. http://dx.doi.org/10.3201/eid1207.051504
5. Svensson K, Larsson P, Johansson D, Byström M, Forsman M, Johansson A. Evolution of subspecies of *Francisella tularensis.* J Bacteriol. 2005;187:3903–8. http://dx.doi.org/10.1128/JB.187.11. 3903-3908.2005
6. Pandya GA, Holmes MH, Petersen JM, Pradhan S, Karamycheva SA, Wolcott MJ, et al. Whole genome single nucleotide polymorphism based phylogeny of *Francisella tularensis* and its application to the development of a strain typing assay. BMC Microbiol. 2009;9:213. http://dx.doi.org/10.1186/1471-2180-9-213
7. Svensson K, Granberg M, Karlsson L, Neubauerova V, Forsman M, Johansson A. A real-time PCR array for hierarchical identification of *Francisella* isolates. PLoS ONE. 2009;4:e8360. http://dx.doi.org/10.1371/journal.pone.0008360

8. Vogler AJ, Birdsell D, Price LB, Bowers JR, Beckstrom-Sternberg SM, Auerbach RK, et al. Phylogeography of *Francisella tularensis*: global expansion of a highly fit clone. J Bacteriol. 2009;191:2474–84. http://dx.doi.org/10.1128/JB.01786-08
9. Keim P, Johansson A, Wagner DM. Molecular epidemiology, evolution, and ecology of *Francisella.* Ann N Y Acad Sci. 2007;1105:30–66. http://dx.doi.org/10.1196/annals.1409.011
10. Kugeler KJ, Mead PS, Janusz AM, Staples JE, Kubota KA, Chalcraft LG, et al. Molecular epidemiology of *Francisella tularensis* in the United States. Clin Infect Dis. 2009;48:863–70. http://dx.doi.org/10.1086/597261
11. Molins CR, Delorey MJ, Yockey BM, Young JW, Sheldon SW, Reese SM, et al. Virulence differences among *Francisella tularensis* subsp. *tularensis* clades in mice. PLoS ONE. 2010;5:e10205. http://dx.doi.org/10.1371/journal.pone.0010205
12. Twine SM, Shen H, Kelly JF, Chen W, Sjostedt A, Conlan JW. Virulence comparison in mice of distinct isolates of type A *Francisella tularensis*. Microb Pathog. 2006;40:133–8. http://dx.doi.org/10.1016/j.micpath.2005.12.004
13. Larsson P, Oyston PC, Chain P, Chu MC, Duffield M, Fuxelius HH, et al. The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nat Genet. 2005;37:153–9. http://dx.doi.org/10.1038/ng1499
14. Gyuranecz M, Birdsell DN, Splettstoesser W, Seibold E, Beckstrom-Sternberg SM, Makrai L, et al. Phylogeography of *Francisella tularensis* subsp. *holarctica*, Europe. Emerg Infect Dis. 2012;18:290–3. http://dx.doi.org/10.3201/eid1802.111305
15. Ayres JC, Feemster R. Epidemiology of tularemia in Massachusetts with a review of the literature. N Engl J Med. 1948;238:187–94. http://dx.doi.org/10.1056/NEJM194802052380606

Address for correspondence: David M. Wagner, Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, AZ 86011-4073, USA; email: dave.wagner@nau.edu

Human Infections with *Rickettsia raoultii*, China

Na Jia,[1] Yuan-Chun Zheng,[1] Lan Ma,[1] Qiu-Bo Huo, Xue-Bing Ni, Bao-Gui Jiang, Yan-Li Chu, Rui-Ruo Jiang, Jia-Fu Jiang, and Wu-Chun Cao

We used molecular methods to identify *Rickettsia raoultii* infections in 2 persons in China. These persons had localized rashes around sites of tick bites. *R. raoultii* DNA was detected in 4% of *Dermacentor silvarum* ticks collected in the same area of China and in 1 feeding tick detached from 1 patient.

Rickettsia raoultii was first detected in *Rhipicephalus pumilio* and *Dermacentor nuttalli* ticks from the former Soviet Union in 1999 and initially named RpA4, DnS14, and DnS28 (*1*). In 2008, it was identified as a novel *Rickettsia* species on the basis of its genetic and serologic characteristics (*2*). Since its emergence, *R. raoultii* has been found to be associated with *Dermacentor* ticks throughout Europe (*3*) and in some parts of Asia, including Mongolia (*4*) and China (*5*,*6*).

A *D. marginatus* tick detached from a patient in France with tick-borne lymphadenopathy/*Dermacentor* tick–borne necrosis erythema and lymphadenopathy was positive for *R. raoultii*, suggesting its pathogenicity for humans (*2*). Seven additional *R. raoultii*–infected cases were later diagnosed in France (*7*). We report 2 cases of *R. raoultii* infection in northeastern China.

The Study

On May 15, 2012, a 67-year-old man came to Mudanjiang Forestry Central Hospital in Mudanjiang, China, with an attached tick on his left shoulder. The feeding tick was detached in the outpatient department and subsequently identified as *D. silvarum* by an entomologist. Four days later, a painful erythematous rash developed around the site of the tick bite, and the patient returned to the hospital for treatment. The rash was irregular, round, and ≈1.5 cm in diameter. No other symptoms and signs were found.

On May 21, 2012, a 30-year-old man came to the same hospital with asthenia, anorexia, nausea, and a painful rash on his abdomen. He had been bitten by a tick 4 days before onset of symptoms. Physical examination identified a 20 × 5 cm erythematous rash on his abdomen around a tick-bite eschar. Vesicles were seen surrounding the site of the tick bite. Lymphadenopathy was not observed.

Routine laboratory and hematologic assays for blood and serum samples from both patients showed standard results. Because rashes were found during physical examinations and infection with spotted fever group rickettsia was suspected, oral doxycycline (100 mg, twice a day for 2 weeks) was then prescribed. The infections of the 2 patients were successfully treated at home and did not recur.

DNA was extracted from anticoagulated blood samples by using a Blood DNA Extraction Kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. PCRs specific for the conserved citrate synthase gene (*glt*A) and spotted fever group–restricted outer membrane protein A gene (*omp*A) were conducted as described (*8*) and followed by sequencing. Both partial *gltA* (341 bp) and *ompA* (325 bp) sequences from the 2 patients were identical to each other and to those of *R. raoultii* (Figure).

PCR results for other tick-borne pathogens, including *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis, Babesia* spp., *Francisella tularensis*, and *Borrelia burgorferi* sensu lato, were negative. Serum specimens were tested by indirect immunofluorescence assay for IgM and IgG against *R. heilongjiangensis*, which was a known *Rickettsia* species circulating in the study area (*9*). IgM titers of 64 and 128, respectively, were observed in serum samples obtained 3 days after disease onset from both patients. The IgG titer was 64 for both patients.

A feeding *D. silvarum* tick detached from the first patient was positive for rickettsial *glt*A and *omp*A by PCR. Sequences were identical to those amplified from the 2 patients (Figure). We used primers CS2d-CSend and Rr190.70-Rr190.602 to amplify longer fragments of *glt*A (1,150 bp) and *omp*A (530 bp), respectively (*8*). Analyses showed that these sequences were identical to corresponding sequences of *R. raoultii* identified in a tick detached from a French patient (*2*).

To identify local natural foci, host-seeking ticks were collected by dragging a fabric flag over vegetation in the area where the 2 patients lived. An entomologist identified all ticks by morphologic features to the species level and developmental stage. DNA was extracted from ticks by using a Tissue DNA Extract Kit (Tiangen Biotechnique Inc., Beijing, China) following the manufacturer's instructions. PCR was performed to amplify rickettsial *glt*A and *omp*A fragments, and sequencing was performed (*8*). *R. raoultii* was identified in 3 (4%) of 75 adult *D. silvarum* ticks and 0 of 453 *Ixodes persulcatus* ticks tested (Figure).

Author affiliations: Beijing Institute of Microbiology and Epidemiology, Beijing, China (N. Jia, L. Ma, W.-C. Cao); Mudanjiang Forestry Central Hospital, Mudanjiang, China (Y.-C. Zheng, Q.-B. Huo, Y.-L. Chu); and State Key Laboratory of Pathogens and Biosecurity, Beijing (X.-B. Ni, B.-G. Jiang, R.-R. Jiang, J.-F. Jiang)

DOI: http://dx.doi.org/10.3201/eid2005.130995

[1]These authors contributed equally to this article.

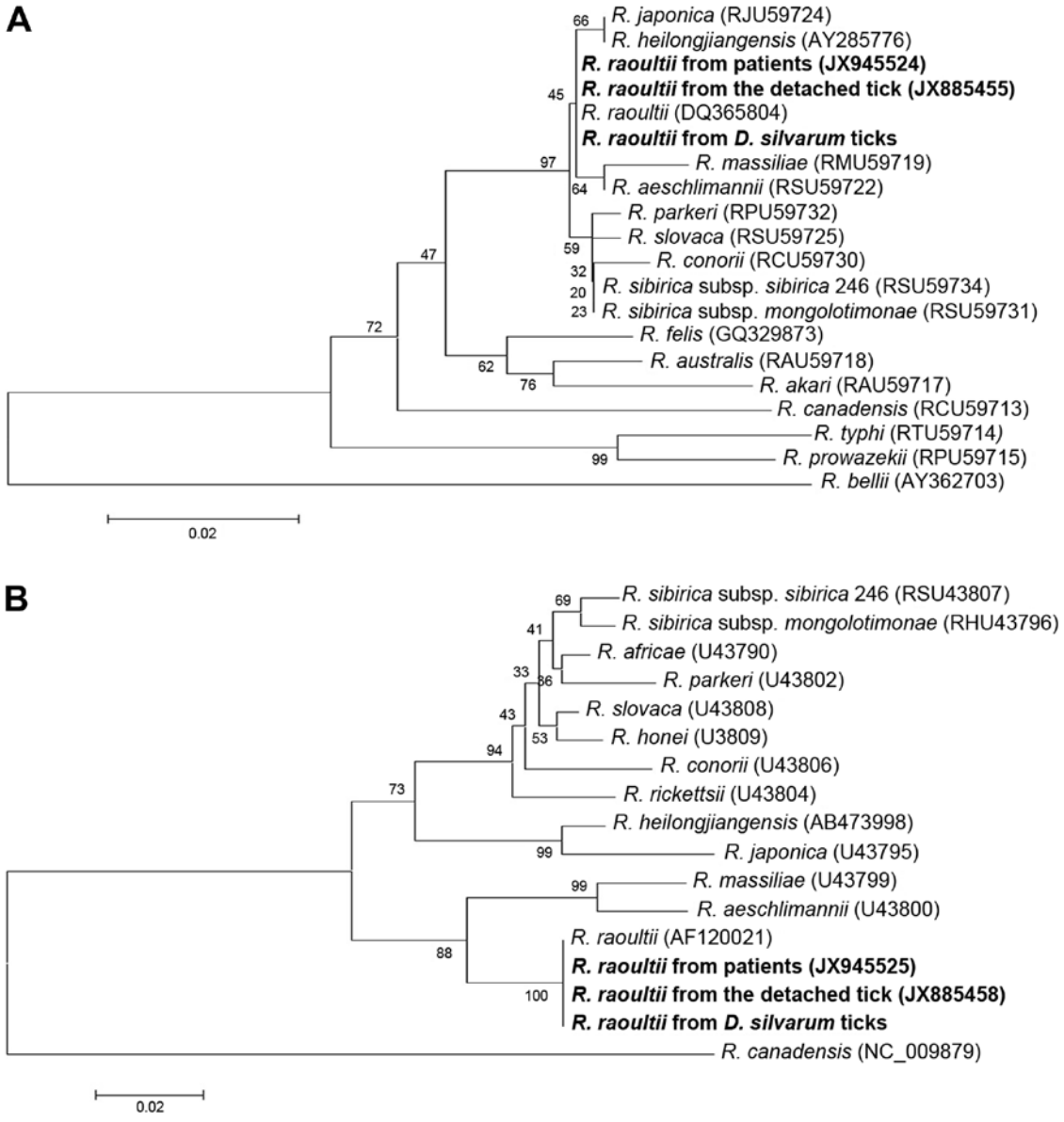


Figure. Phylogenetic analysis of spotted fever group *Rickettsia* species, China, based on A) partial (341 bp) citrate synthase gene and B) partial (325 bp) 190-kDa outer membrane protein gene. Trees were obtained by using the neighbor-joining method, distances were calculated by using Kimura 2-parameter analysis, and analysis was conducted by using Mega 5.0 software (www.megasoftware.net/). Nucleotide sequences determined in this study are indicated in boldface. Percentage of replicate trees in which associated taxa clustered in the bootstrap test (1,000 replicates) is shown to the left of each branch. Trees are drawn to scale, and branch lengths have the same units as evolutionary distances used to infer the phylogenetic tree. GenBank accession numbers of sequences used in phylogenetic analysis are indicated in parentheses. Scale bars indicate nucleotide substitutions per site.

Conclusions

Eight cases of human infection with *R. raoultii* have been reported in France. All patients had tick-borne lymphadenopathy/*Dermacentor* tick–borne necrosis erythema and lymphadenopathy, which was defined as the association of a tick bite, an inoculation eschar on the scalp, and cervical lymphadenopathies (*10*).

Although the 2 patients in China had only painful rashes around the sites of tick bites, rather than tick-borne lymphadenopathy/*Dermacentor* tick–borne necrosis erythema and lymphadenopathy, the difference in clinical features might be the result of location of tick bites. Tick-borne lymphadenopathy/*Dermacentor* tick–borne necrosis erythema and lymphadenopathy is usually observed in

patients after tick bites on the scalp, regardless of whether the disease is caused by *R. slovaca*, *R. rioja*, or *R. raoultii* (*2*,*7*,*11*). The 2 patients had tick bites on abdomen and shoulder, respectively, but no cervical lymphadenopathy developed.

Isolation in cell cultures is the ultimate diagnostic method for rickettsial infections, but it is available only in biosafety level 3 laboratories (*9*). However, serologic tests are useful tools, and indirect immunofluorescence assay is considered the reference method. PCR, followed by sequencing is mainly used to detect and identify *Rickettsia* species and is also an effective diagnostic method (*9*,*12*).

Of the 8 cases of human infection with *R. raoultii* in France, 4 were identified by PCR amplification from ticks detached from patients, and 4 were identified by rickettsial-specific serologic assays (*2*,*7*). In contrast, we identified 2 cases of human infection with *R. raoultii* in China by molecular detection and sequence determination of the pathogen in blood samples and from a tick detached from 1 of the patients. Although only acute-phase (3 days after disease onset) serum samples were available, the presence of IgM and IgG against *R. heilongjiangensis* supported the diagnoses (*12*).

R. raoultii has been detected in *Dermacentor* ticks, such as *D. silvarum, D. nuttalli, D. reticulatus*, and *D. marginatus* (*1*,*2*), and infection rates in Europe range from 2% to 80% (*13*). *R. raoultii* was also found in 4% of *D. silvarum* ticks collected in the same area of China. We previously identified a novel rickettsial agent identical to DnS14 from *D. silvarum* ticks, which was later named *R. raoultii*, in Jilin Province neighboring this study site (*6*). *D. silvarum* ticks are distributed over a wide area in northeastern China, parasitize many domestic and wild animals, and readily feed on humans as alternate hosts. Extended investigation and tick surveillance are required to understand the distribution of *R. raoultii* in this region.

The current recommended therapeutic regimen for rickettsiosis is administration of tetracyclines; doxycycline is the preferred agent because of its dosage (twice a day) and better patient tolerance. The 2 patients received doxycycline treatment at home and had uneventful recoveries. These findings indicate that doxycycline should be administrated as soon as possible to patients who have been exposed to ticks and have clinical manifestations suggestive of rickettsiosis.

Acknowledgments

We thank Yu-Dong Song, Nan-Nan Yao, and doctors and nurses in the Department of Tick-borne Infectious Disease in Mudanjiang Forestry Central Hospital for help with sample collection, and Bo-Hai Wen and Xi-Le Wang for technical support with serologic testing.

This study was supported by the Natural Science Foundation of China (81290344 and 81130086)

Dr Jia is a scientist at the Beijing Institute of Microbiology and Epidemiology, Beijing, China. Her research interest is tick-borne diseases.

References

1. Rydkina E, Roux V, Fetisova N, Rudakov N, Gafarova M, Tarasevich I, et al. New rickettsiae in ticks collected in territories of the former Soviet Union. Emerg Infect Dis. 1999;5:811–4. http://dx.doi.org/10.3201/eid0506.990612
2. Mediannikov O, Matsumoto K, Samoylenko I, Drancourt M, Roux V, Rydkina E, et al. *Rickettsia raoultii* sp. nov., a spotted fever group rickettsia associated with *Dermacentor* ticks in Europe and Russia. Int J Syst Evol Microbiol. 2008;58:1635–9. http://dx.doi.org/10.1099/ijs.0.64952-0
3. Spitalská E, Stefanidesova K, Kocianova E, Boldis V. *Rickettsia slovaca* and *Rickettsia raoultii* in *Dermacentor marginatus* and *Dermacentor reticulatus* ticks from Slovak Republic. Exp Appl Acarol. 2012;57:189–97. http://dx.doi.org/10.1007/s10493-012-9539-8
4. Speck S, Derschuma H, Damdindorj T, Dashdavaa O, Jiang J, Kaysser PB, et al. *Rickettsia raoultii,* the predominant *Rickettsia* found in Mongolian *Dermacentor nuttalli.* Ticks Tick Borne Dis. 2012;3:227–31. http://dx.doi.org/10.1016/j.ttbdis.2012.04.001
5. Tian ZC, Liu GY, Shen H, Xie JR, Luo J, Tian MY. First report on the occurrence of *Rickettsia slovaca* and *Rickettsia raoultii* in *Dermacentor silvarum* in China. Parasit Vectors. 2012;5:19. http://dx.doi.org/10.1186/1756-3305-5-19
6. Cao WC, Zhan L, De Vals SJ, Wen BH, Yang H, Richardus JH, et al. Molecular detection of spotted fever group *Rickettsia* in *Dermacentor silvarum* from a forest area of northeastern China. J Med Entomol. 2008;45:741–4. http://dx.doi.org/10.1603/0022-2585(2008)45[741:MDOSFG]2.0.CO;2
7. Parola P, Rovery C, Rolain JM, Brouqui P, Davoust B, Raoult D. *Rickettsia slovaca* and *R. raoultii* in tick-borne rickettsioses. Emerg Infect Dis. 2009;15:1105–8. http://dx.doi.org/10.3201/eid1507.081449
8. Jia N, Zheng YC, Jiang JF, Ma L, Cao WC. Human infection with *Candidatus Rickettsia tarasevichiae.* N Engl J Med. 2013;369:1178–80. http://dx.doi.org/10.1056/NEJMc1303004
9. La Scola B, Raoult D. Laboratory diagnosis of rickettsioses: current approaches to the diagnosis of old and new rickettsial diseases. J Clin Microbiol. 1997;35:2715–27.
10. Raoult D, Lakos A, Fenollar F, Beytout J, Brouqui P, Fournier PE. Spotless rickettsiosis caused by *Rickettsia slovaca* and associated with *Dermacentor* ticks. Clin Infect Dis. 2002;34:1331–6. http://dx.doi.org/10.1086/340100
11. Oteo JA, Portillo A. Tick-borne rickettsioses in Europe. Ticks Tick Borne Dis. 2012;3:271–8. http://dx.doi.org/10.1016/j.ttbdis.2012.10.035
12. Centers for Disease Control and Prevention. Fatal cases of Rocky Mountain spotted fever in family clusters—three states, 2003. MMWR Morb Mortal Wkly Rep. 2004;53:407–10.
13. Silaghi C, Dietmar H, Thiel C, Pfister K, Pfeffer M. Spotted fever group rickettsiae in ticks, Germany. Emerg Infect Dis. 2011;17:890–2. http://dx.doi.org/10.3201/eid1705.101445

Address for correspondence: Wu-Chun Cao, Beijing Institute of Microbiology and Epidemiology, 20 Dong-Da St, Fengtai District, Beijing 100071, China; email: caowc@bmi.ac.cn

Extensively Drug-Resistant *Streptococcus pneumoniae*, South Korea, 2011–2012

Sun Young Cho,[1] Jin Yang Baek,[1] Cheol-In Kang, So Hyun Kim, Young Eun Ha, Doo Ryeon Chung, Nam Yong Lee, Kyong Ran Peck, and Jae-Hoon Song

To better understand extensively drug resistant *Streptococcus pneumoniae*, we assessed clinical and microbiological characteristics of 5 extensively drug-resistant pneumococcal isolates. We concluded that long-term care facility residents who had undergone tracheostomy might be reservoirs of these pneumococci; 13- and 23-valent pneumococcal vaccines should be considered for high-risk persons; and antimicrobial drugs should be used judiciously.

During the past 2 decades, multidrug-resistant *Streptococcus pneumoniae* has spread worldwide (*1*,*2*). Recently we reported a case of bacteremic pneumonia caused by extensively drug-resistant (XDR) *S. pneumoniae* (*3*). Five additional XDR pneumococcal isolates subsequently were identified in our hospital in South Korea. In an attempt to better understand the epidemiologic and clinical aspects of XDR *S. pneumoniae*, we investigated clinical and microbiological characteristics of these cases of XDR *S. pneumoniae*.

The Study

We reviewed the database of the clinical microbiology laboratory at Samsung Medical Center (SMC, Seoul, South Korea) for XDR *S. pneumoniae* isolates obtained during 2011–2012. XDR *S. pneumoniae* was defined as nonsusceptibility to at least 1 agent in all antibacterial drug categories except vancomycin and linezolid.

Among the 510 *S. pneumoniae* isolates (319 in 2011 and 191 in 2012), we identified 5 XDR pneumococcal isolates from 5 (1.2%) patients. The following data were obtained for the patients: age, sex, date of isolation, prior hospitalization, residence in long-term care facilities (LTCFs), underlying diseases, site of bacterial isolation, status of bacterial isolation (infection or colonization), prior prophylaxis and therapy with antibacterial drugs, and outcome.

In vitro antimicrobial susceptibility tests of pneumococcal isolates were retested by the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines (*4*). Antimicrobial classes tested included penicillins (penicillin), cephalosporins (ceftriaxone), macrolides (erythromycin, clarithromycin), quinolones (levofloxacin), clindamycin, trimethoprim/sulfamethoxazole, carbapenems (imipenem), tetracyclines (tetracycline, tigecycline), glycopeptides (vancomycin), and linezolid. Interpretive criteria for susceptibility were those indicated in a Clinical and Laboratory Standards Institute document (*5*). Serotypes of *S. pneumoniae* were determined by the capsular puellung method with commercial antiserum (Statens Serum Institut, Copenhagen, Denmark) as recommended by the manufacturer. To investigate the molecular characteristics of XDR *S. pneumoniae*, we performed multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) as described (*6*,*7*). Also, all isolates were subjected to PCR to detect quinolone resistance–determining regions and macrolide resistance genes as described (*8*,*9*).

Four of 5 XDR *S. pneumoniae* isolates had been isolated from respiratory tract specimens (e.g., sputum), and 1 isolate had been recovered from blood (Table 1, Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1371-T1.htm). Mean age (± SD) of patients was 71.8 (± 16.9) years. Three patients were admitted from 3 different LTCFs, and 2 patients were referred to our hospital from other acute care hospitals. The most common underlying diseases were neurologic disorders, such as cerebrovascular disease and motor neuron disease (3 patients), followed by diabetes mellitus (2 patients) and solid tumor (1 patient) (1 patient had both motor neuron disease and diabetes mellitus). Four patients underwent tracheostomy because of respiratory problems, such as neurologic disease and progression of underlying diseases, and had multiple episodes of aspiration pneumonia. All patients had received antibacterial drug therapy within the past 3 months. The most frequently used antibacterial drugs were fluoroquinolones (4 patients) and piperacillin–tazobactam (3 patients). Four of the 5 patients did not have clinical evidence of infection with XDR *S. pneumoniae*; bacteremia from this pathogen developed in the remaining patient. The patient with bacteremia was admitted to the emergency department of SMC with fever. Three weeks before admission, nephrotic syndrome had been diagnosed, and the patient had received immunosuppressant therapy with methylprednisolone and cyclophosphamide. Soon after admission, septic shock developed, and ciprofloxacin was administered because urinary tract

Author affiliations: Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea (S.Y. Cho, C.-I. Kang, Y.E. Ha, D.R. Chung, N.Y. Lee, K.R. Peck, J.-H. Song); and Asia Pacific Foundation for Infectious Diseases, Seoul (J.Y. Baek, S.H. Kim, J.-H. Song)

DOI: http://dx.doi.org/10.3201/eid2005.131371

[1]These authors contributed equally to this article.

Table 2. Antimicrobial susceptibilities of 5 extensively drug-resistant *Streptococcus pneumoniae* isolates, Seoul, South Korea, 2011–2012*

Strain no.	MIC, μg/mL										
	LZD	TGC	VAN	PEN	TMP/SXT	CLI	CRO	LVX	ERY	CLR	TET	IPM
SMC1101–114	0.5	<0.03	0.5	8	32/608	>32	16	16	64	16	32	2
SMC1101–127	0.5	<0.03	0.25	8	32/608	>32	64	16	128	>32	32	2
SMC1103–146	0.5	<0.03	0.25	8	32/608	>32	64	16	128	>32	32	2
SMC1105–235	0.5	<0.03	0.25	8	32/608	>32	32	16	128	>32	32	2
SMC1108–054	0.5	<0.03	0.5	8	32/608	>32	64	16	128	>32	16	2

*LZD, linezolid; TGC, tigecycline; VAN, vancomycin; PEN, penicillin; TMP/SXT, trimethoprim/sulfamethoxazole; CLI, clindamycin; CRO, ceftriaxone; LVX, levofloxacin; ERY, erythromycin; CLR, clarithromycin; TET, tetracycline; IPM, imipenem; SMC, Samsung Medical Center.

infection was suspected. On hospital day 4, after the blood culture results showed XDR *S. pneumoniae*, vancomycin was started. Despite the administration of vancomycin for 7 days, the patient died from progression of septic shock. Because she had difficulty in expectorating sputum, we could not obtain adequate sputum samples despite sputum induction. However, given the clinical symptoms consistent with pneumonia and a chest radiograph that demonstrated pulmonary infiltrate, the most likely source of bacteremia appears to be pneumonia caused by *S. pneumoniae*.

The 5 pneumococcal isolates reported in this study were nonsusceptible to all tested antimicrobial agents except tigecycline, vancomycin, and linezolid (Table 2). The serotypes of isolates were 11A (3 isolates) and 13/28 (1 isolates); 1 isolate was not typeable. MLST demonstrated that 4 isolates were sequence type (ST) 8279, a double-locus variant of ST156 closely related to the pneumococcal Spain9V-3 international clone, and 1 isolate was ST3598. PFGE patterns showed close genetic relatedness among 3 isolates and 2 isolates (94.7% and 84.2% genetic relatedness, respectively) (Figure). However, SMC 1205–093, reported in 2012, had a different PFGE pattern from the other 5 isolates. Sequence analysis of quinolone resistance–determining regions revealed the same mutation pattern in these 5 isolates: Ser81-Phe in *gyrA*, Lys137-Asn in *parC*, and Ile460-Val in *parE*. In addition, as a macrolide resistance determinant, only *erm*(B) gene was detected by PCR in all isolates.

Conclusions

We reassessed isolates obtained during 2011–2012 and identified 5 pneumococcal isolates that were not susceptible to at least 1 agent in all antimicrobial classes tested (penicillins, cephalosporins, macrolides, quinolones, clindamycin, tetracyclines, trimethoprim/sulfamethoxazole and carbapenems) except vancomycin and linezolid. Of the 5 patients, 1 with bacteremia died despite treatment.

Our findings have several clinical implications. First, the cases reported here showed that LTCF residents who had undergone tracheostomy might be a reservoir of XDR pneumococci. Also, our data documented a genetic relationship between XDR pneumococcal isolates shown by MLST and PFGE, which suggests that specific serotypes and resistant clones are spreading within certain LTCFs. Considering the characteristics of LTCF residents, the spread of XDR *S. pneumoniae* among these patients can lead to considerable illness and to death.

Second, this study indicated that 3 of the 5 XDR isolates were serotype 11A, which were included in 23-valent pneumococcal polysaccharide vaccine (PPV23) but not in the 7-valent pneumococcal conjugate vaccine (PCV7) and the 13-valent pneumococcal conjugate vaccine (PCV13). Although we were unable to determine the status of pneumococcal vaccination of the patients reported here, given the very low rates of PPV23 vaccination in Korea (<5%), these patients were likely to be unvaccinated (*10*). Among pneumococcal isolates collected from patients

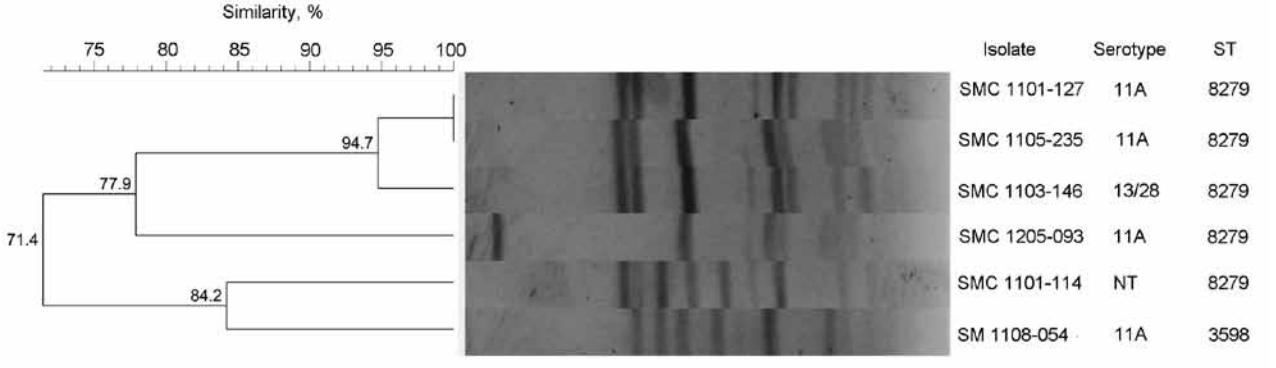


Figure. Dendrogram of pulsed-field gel electrophoresis patterns showing the genetic relatedness of extensively drug-resistant pneumococcal isolates from patients in South Korea, 2011–2012 (including SMC 1205–093, previously reported in 2012). The corresponding serotype and sequence type (ST) for each isolate are listed on the right side of the dendrogram. SMC, Samsung Medical Center.

with respiratory tract infections in South Korea, the prevalence rate of serotype 11A was 7.6%, and these serotype 11A isolates showed a high prevalence of multidrug-resistance (65.2%) (*11*). In addition, after the introduction of PCV7 in Korea, nonvaccine serotypes (7C, 11A, 15A, 16F, and 23A) have increased in levofloxacin-nonsusceptible pneumococcal isolates (*12*). PCV13 was approved for all adults ≥50 years of age in 2012 and is now widely used in South Korea. If PCV13 is routinely used instead of PPV23 for adults, non-PCV13 serotypes with multidrug resistance potentially could emerge. Therefore, in adults at risk for MDR pneumococcal infection, including previous use of antibacterial drugs, LTCF residence, and multiple comorbidities, administration of 2 pneumococcal vaccines (PCV13 and PPV23) should be considered.

Third, antibacterial drugs should be used judiciously. In particular, given the increasing evidence that prior use of fluoroquinolones may be a major risk factor for fluoroquinolone resistance among pneumococci, the use of fluoroquinolones should be restricted to patients at increased risk for MDR pneumococcal infection (*13*,*14*).

Although still rare, the emergence of XDR pneumococci has become challenging for clinicians and a real threat to public health. More information about the emergence and spread of this XDR strain is necessary to prevent its spread, and continuous surveillance of XDR *S. pneumoniae* is strongly warranted.

This study was supported by the Basic Research Program through the National Research Foundation of Korea, funded by the Ministry of Education, Science and Technology (no. 2010-0021572). Bacterial isolates were obtained from the Asian Bacterial Bank of the Asia Pacific Foundation for Infectious Diseases. No competing financial interest exists.

Dr Cho is an infectious disease physician at Samsung Medical Center, Seoul, South Korea. Her main interests are medical bacteriology and infectious disease epidemiology.

References

1. Mera RM, Miller LA, Daniels JJ, Weil JG, White AR. Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States over a 10-year period: Alexander project. Diagn Microbiol Infect Dis. 2005;51:195–200. http://dx.doi.org/10.1016/j.diagmicrobio.2004.10.009
2. Kim SH, Song JH, Chung DR, Thamlikitkul V, Yang Y, Wang H, et al. Changing trends in antimicrobial resistance and serotypes of *Streptococcus pneumoniae* isolates in Asian countries: an Asian Network for Surveillance of Resistant Pathogens (ANSORP) study. Antimicrob Agents Chemother. 2012;56:1418–26. http://dx.doi.org/10.1128/AAC.05658-11
3. Kang CI, Baek JY, Jeon K, Kim SH, Chung DR, Peck KR, et al. Bacteremic pneumonia caused by extensively drug-resistant *Streptococcus pneumoniae.* J Clin Microbiol. 2012;50:4175–7. http://dx.doi.org/10.1128/JCM.01642-12
4. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M07–A8. Wayne (PA): The Institute; 2009.
5. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twenty-first informational supplement. Approved standard M100–S21. Wayne (PA): The Institute; 2011.
6. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology. 1998;144:3049–60. http://dx.doi.org/10.1099/00221287-144-11-3049
7. Matushek MG, Bonten MJ, Hayden MK. Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. J Clin Microbiol. 1996;34:2598–600.
8. Ko KS, Song JH. Evolution of erythromycin-resistant *Streptococcus pneumoniae* from Asian countries that contains *erm*(B) and *mef*(A) genes. J Infect Dis. 2004;190:739–47. http://dx.doi.org/10.1086/422156
9. Oh WS, Suh JY, Song JH, Ko KS, Jung SI, Peck KR, et al. Fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae* from Asian countries: ANSORP study. Microb Drug Resist. 2004;10:37–42. http://dx.doi.org/10.1089/107662904323047781
10. Song JY, Cheong HJ, Heo JY, Noh JY, Seo YB, Kim IS, et al. Outpatient-based pneumococcal vaccine campaign and survey of perceptions about pneumococcal vaccination in patients and doctors. Yonsei Med J. 2013;54:469–75. http://dx.doi.org/10.3349/ymj.2013.54.2.469
11. Lee S, Lee K, Kang Y, Bae S. Prevalence of serotype and multidrug-resistance of *Streptococcus pneumoniae* respiratory tract isolates in 265 adults and 36 children in Korea, 2002–2005. Microb Drug Resist. 2010;16:135–42. http://dx.doi.org/10.1089/mdr.2009.0114
12. Lee S, Kim SH, Park M, Bae S. High prevalence of multiresistance in levofloxacin-nonsusceptible *Streptococcus pneumoniae* isolates in Korea. Diagn Microbiol Infect Dis. 2013;76:227–31. http://dx.doi.org/10.1016/j.diagmicrobio.2013.02.032
13. Ho PL, Tse WS, Tsang KW, Kwok TK, Ng TK, Cheng VC, et al. Risk factors for acquisition of levofloxacin-resistant *Streptococcus pneumoniae*: a case-control study. Clin Infect Dis. 2001;32:701–7. http://dx.doi.org/10.1086/319222
14. von Gottberg A, Klugman KP, Cohen C, Wolter N, de Gouvenia L, du Plessis M, et al. Emergence of levofloxacin-non-susceptible *Streptococcus pneumoniae* and treatment for multidrug-resistant tuberculosis in children in South Africa: a cohort observational surveillance study. Lancet. 2008;371:1108–13. http://dx.doi.org/10.1016/S0140-6736(08)60350-5

Address for correspondence: Cheol-In Kang, Division of Infectious Diseases, Samsung Medical Center, Sungkyunkwan University School of Medicine, Irwon-ro 81, Gangnam-gu, Seoul, 135-710, South Korea; email: collacin@hotmail.com

Role of Transportation in Spread of Porcine Epidemic Diarrhea Virus Infection, United States

James Lowe, Phillip Gauger, Karen Harmon, Jianqiang Zhang, Joseph Connor, Paul Yeske, Timothy Loula, Ian Levis, Luc Dufresne, and Rodger Main

After porcine epidemic diarrhea virus (PEDV) was detected in the United States in 2013, we tested environmental samples from trailers in which pigs had been transported. PEDV was found in 5.2% of trailers not contaminated at arrival, , suggesting that the transport process is a source of transmission if adequate hygiene measures are not implemented.

Porcine epidemic diarrhea virus (PEDV) was detected in herds of pigs in the United States during April 2013 (*1*). PEDV is a member of the *Cornaviridae* family that produces a malabsorptive diarrhea secondary to atrophy of the small intestinal villi (*2*). Initial clinical cases were detected in herds in Indiana and Iowa during May 2013. The virus spread rapidly across large geographic regions; 218 cases of infection were identified in 16 states during the first 9 weeks of the outbreak (*3*). Subsequent testing of historical samples collected during the week of April 15, 2013 identified the index herd in Ohio (*3*). Veterinarians became concerned about the role that facilities where pigs are harvested for processing into food and the transportation equipment used to move pigs from farms to those facilities were playing in the spread of PEDV. These concerns were based on evidence that equipment used to transport live pigs transmits another enteric coronavirus, transmissible gastroenteritis virus, between sites in the United States (J.F. Lowe, unpub. data).

Author affiliations: University of Illinois, Urbana, Illinois, USA (J. Lowe); Iowa State University, Ames, Iowa, USA (P. Gauger, K Harmon, J. Zhang, R. Main); Carthage Veterinary Service, Ltd., Carthage, Illinois, USA (J. Connor); Swine Vet Center, P.A., St. Peter, MN, USA (P. Yeske, T. Loula); Iowa Select Farms, Iowa Falls, Iowa, USA (I. Levis); and Seaboard Foods, Kansas City, Missouri, USA (L. Dufresne)

DOI: http://dx.doi.org/10.3201/eid2005.131628

Pigs are commonly transported to harvest facilities in vehicles that have not been cleaned and disinfected between loads. Implementation of "all in–all out" sites, which are sites in which pigs are grown and all pigs in a group are removed before arrival of the next group, limits the spread of disease introduced by transport vehicles. In many cases, the risks and associated costs of disease introduced late in the growing period are thought to be less than the cost of cleaning and disinfecting vehicles. Transport vehicles are often shared by different pig owners, enabling the spread of disease across large regions.

The Study

The objective of this study was to assess the risks that harvest facilities and transport vehicles engendered in promoting the initial outbreak of a novel disease organism by estimating the incidence of trailer contamination with PEDV during the unloading process at harvest facilities. Environmental samples were collected from 575 livestock trailers before and after pigs were unloaded into holding pens, or lairages, at 6 harvest facilities (83–102 trailers per facility) located in the central United States. Samples were collected during a period of 2–3 days at each facility during June 14–20, 2013. For each trailer, the following information was collected: transport company and trailer identification, time of unloading, dock used, whether the truck driver stepped on the dock, and whether facility personnel entered the trailer. Sample collection consisted of rubbing a phosphate-buffered saline–moistened pad (Swiffer, Procter & Gamble, Cincinnati, OH, USA) over an ≈900 cm^2 area of the trailer floor, 15 cm from the rear door. The pad was placed in a sterile bag (Whirl-Pac, NASCO, Fort Atkinson, WI, USA) and the liquid was collected by applying manual pressure. The liquid was transferred to a sterile tube (14mL Falcon Tube, Fisher Scientific, Chicago, IL, USA), immediately placed on ice, and maintained at 4°C during transport to the Iowa State University Veterinary diagnostic laboratory. New latex gloves were worn for each sample collection to minimize the risk for cross-contamination.

RNA extraction was performed with 100 mL of each environmental sample by using the MagMAX Viral RNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) and a Kingfisher 96 instrument (Thermo Scientific, Waltham, MA, USA) and Kingfisher program AM_1836_DW_HV_v3 provided by the manufacturer of the viral extraction kits. Viral RNA was eluted into 90 μL of buffer. Real-time reverse transcription PCR (rRT-PCR) was performed on nucleic acid extracts by using the Path-ID Multiplex One-Step RT-PCR Kit (Life Technologies) according to the manufacturer's recommendations. Primers and probe targeting conserved regions of the PEDV

nucleocapsid protein gene were as described (*4*) with modifications specific to the sequence isolated in North America deposited in GenBank (accession no. KF272920). The forward primer sequence was 5′-CGCAAAGACTGAACCCACTAACCT-3′, the reverse primer sequence was 5′-TTGCCTCTGTTGTTACTTGGAGAT-3′, and the probe sequence was 5′-TGTTGCCATTACCACGACTCCTGC-3′. Sequences were labeled by using the FAM/ZEN/3′ Iowa Black detector (Integrated DNA Technologies, Coralville, IA, USA). All rRT-PCR reactions were conducted on an ABI 7500 Fast (Applied Biosystems, Foster City, CA, USA) and results analyzed by system software. Samples were tested separately from routine diagnostic samples in the laboratory to minimize risks for cross-contamination.

Before unloading, 38 (6.6%) of the 575 trailers were contaminated with PEDV. The proportion of contaminated trailers ranged from 2% to 14.6% among the 6 harvest facilities; the facility level median was 5.0%. Of the remaining 537, 28 (5.2%) that were not contaminated at arrival were contaminated in the unloading process (Table).

Of the 38 trailers that were contaminated on arrival, environmental samples from 13 (34.2%) were negative for PEDV after unloading. Environmental samples from these 13 trailers tended to have higher cycle threshold values than those from the 25 trailers that were positive before and after unloading: 32.3 versus 30.6, respectively. This result suggests that the pigs transported to the harvest facility on the 13 trailers may not have been shedding PEDV, but instead, the trailers had been contaminated by previous loads of pigs, so viral quantities in the trailer were low or at the limit of detection.

Contamination during unloading occurred at a higher rate if harvest facility staff entered the trailer (OR 4.15, 95% CI 1.27–13.54) or if unloading occurred immediately after unloading another trailer that was found to be contaminated (OR 3.35, 95% CI 1.22–9.18). Facilities in which more PEDV was identified in truck trailers on arrival had a higher overall incidence of contamination. This was measured by multiplying the prevalence of contamination at arrival by the inverse of the cycle threshold value from trailers contaminated at arrival ($R^2 = 0.32$, $p = 0.01$). All drivers stepped into the harvest facility at least once, leading to a high rate of contact between drivers, the trailers, and the harvest facility.

Table. Status of environmental samples from pig transport trailers during an outbreak of porcine epidemic diarrhea virus infection, Midwestern United States, June 2013*

PCR status after unloading	PCR test status before unloading		
	Positive	Negative	Total
Positive	25 (4.3)	28 (4.9)	53 (9.2)
Negative	13 (2.3)	509 (88.5)	522 (90.8)
Total	38 (6.6)	537 (93.4)	575

*Values represent the number of trailers (% total) in each group. Samples were gathered from 6 pig harvest facilities.

Conclusions

Harvest facilities serve as a source of contact between many swine farms with different health statuses. This study suggests that collection points, such as harvest facilities and livestock auction markets, can be an efficient source of contamination of transport vehicles that return to pig farms and likely played a role in rapidly disseminating PEDV across vast geographic regions shortly after PEDV was first identified in the United States. These data also suggest that the contamination of transport vehicles leaving the harvest facilities increased as the prevalence of PEDV–positive transport vehicles and virus load coming into the facility increased.

The results of this study suggest that proactive disease control measures should include improved sanitation, hygiene, and segregation practices at collection points to limit the spread of the agent early in the outbreak. Current data suggest that novel agents, such as PEDV, may be present in a country but remain undetected for an extended period. Thus, control measures may be implemented too late to limit the spread of the disease through fomites that are identified, such as, in this instance, contaminated vehicles returning from swine collection points. Simple measures such as limiting contact between drivers and the collection point and requiring drivers to remain on trucks and out of the collection point during the unloading process may have a dramatic effect on limiting the transmission of novel agents. These biosecurity measures are simple but require a coordinated effort between producers, transporters, harvest facility owners, and regulators to achieve effective implementation. This study of PEDV transmission by fomites should serve as an example of the risks that a modern, highly technical animal protein industry may encounter during a novel disease introduction. PEDV's introduction and subsequent spread in the United States should spur action to minimize these risks before a disease that can affect international trade or food safety is introduced.

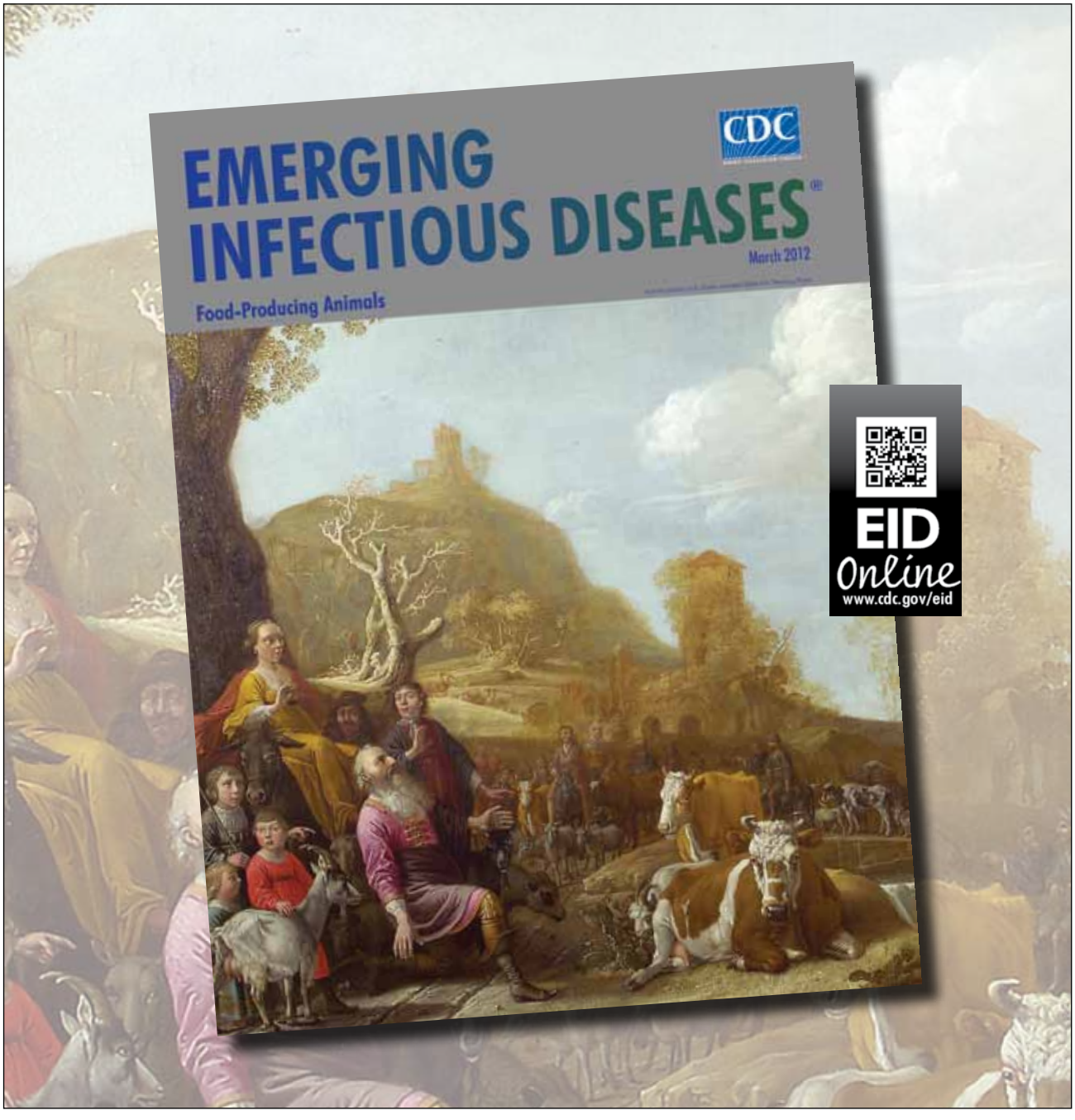
The National Pork Board, the National Pork Producers Council, the American Association of Swine Veterinarians, and the American Association of Swine Veterinarians Foundation funded this project.

Dr Lowe is a clinical instructor in the Department of Veterinary Clinical Medicine, University of Illinois, and is an adjunct faculty member of Kansas State University and the University of Minnesota. He also owns Lowe Consulting Ltd. and Production Animal Consultation, LLC. His research interests include the effects of management on host–pathogen interactions and livestock growth performance.

References

1. Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D, et al. Emergence of porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J Vet Diagn Invest. 2013;25:649–54. http://dx.doi.org/10.1177/1040638713501675
2. Saif LJ, Pensaert MB, Sestak K, Yeo S, and Jung K. Coronaviruses. In: Zimmerman J, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of swine. Ames (IA): Wiley & Sons; 2012. p. 501–24.
3. US Department of Agriculture, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories. Porcine Epidemic Diarrhea virus (PEDv) Testing Data from NAHLN Laboratories: PED/PEDv_weekly_report [cited 2013 Jul 3]; http://www.aasv.org/aasv%20website/Resources/Diseases/PED/PEDv_weekly_report_070313.pdf.
4. Kim SH, Kim IJ, Pyo HM, Tark DS, Song JY, Hyun BH. Multiplex real-time RT-PCR for the simultaneous detection and quantification of transmissible gastroenteritis virus and porcine epidemic diarrhea virus. J Virol Methods. 2007;146:172–7. http://dx.doi.org/10.1016/j.jviromet.2007.06.021

Address for correspondence: James F. Lowe, Department of Veterinary Clinical Medicine, 270 LAC, 1008 W Hazelwood Dr, Urbana, IL 61802, USA; email: jlowe@illinois.edu



Acute Lower Respiratory Tract Infections in Soldiers, South Korea, April 2011–March 2012

Jung Yeon Heo,[1] Ji Eun Lee,[1] Hye Kang Kim, and Kang-Won Choe

During April 2011–March 2012, we retrospectively reviewed medical records for South Korea soldiers to assess the etiology and epidemiology of acute viral lower respiratory tract infections. Adenovirus was the most commonly identified virus (63.2%) and the most common cause of pneumonia (79.3%) and hospitalization (76.6%); 3 soldiers died of adenovirus-related illness.

Respiratory infections are the most common cause for hospitalization of soldiers. In the US military, respiratory infections account for 25%–30% of all hospital admissions for infectious diseases (*1*). Adenovirus is the most common cause of acute respiratory infection in soldiers, particularly among new recruits in basic training camps (*2*,*3*). A study among US military showed that in the early phases of basic training, ≈10% of new recruits were infected with adenovirus, and among recruits with pneumonia, 90% of the cases were caused by adenovirus (*4*). In US military, adenovirus serotypes 4 and 7 have been historically the most common cause of febrile respiratory illness (*5*–*8*). Since 1971, the US military has orally vaccinated new recruits with live adenovirus vaccine; this vaccination has become the primary preventive strategy against respiratory diseases caused by adenovirus (*9*,*10*).

In spring 2006 in South Korea, a high adenovirus prevalence of 61% was found among military recruits with mild respiratory disease (*11*). More recently in South Korea, deaths caused by severe pneumonia were reported among the military, and an outbreak of acute respiratory disease caused by adenovirus occurred in an army training camp (*12*,*13*). We hypothesized that, although there may be some differences in etiologic agents by geographic location, the major cause of acute respiratory disease in South Korean military recruits is most likely adenovirus, as observed in the US military. To describe the viral etiology, clinical features, and epidemiologic characteristics of acute lower respiratory tract infections (LTRIs) among the South Korean military, we retrospectively reviewed the medical records of soldiers who were identified with an acute LTRI.

The Study

The study was conducted during April 2011–March 2012 at the Armed Forces Capital Hospital, a military referral hospital in Seongnam, South Korea. We retrospectively reviewed all medical records with International Classification of Diseases, Tenth Revision, Clinical Modification codes indicating acute LTRI (pneumonia, tracheobronchitis, or bronchiolitis); 622 patient records met the criteria and were reviewed. General characteristics of the study population and the military hospital, as well as the method used for classifying clinical diagnoses of acute LTRI, are available in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/5/13-1692-Techapp1.pdf).

Nasopharyngeal swab specimens were collected from 207 (33.3%) of the 622 patients with an acute LTRI. Within 3 days, the specimens were tested (GClabs, Yongin-si, South Korea) for 12 respiratory viruses by multiplex reverse transcription PCR; methods are described in the online Technical Appendix. Respiratory virus infections were confirmed for 87 (42.0%) patients: adenovirus (55 [63.2%] patients), influenza A virus (26 [29.9%] patients), influenza B virus (4 [4.6%] patients), rhinovirus group A (3 [3.4%] patients), and parainfluenza virus (2 [2.3%] patients). Co-infection with adenovirus and rhinovirus group A was observed in 3 patients. For all confirmed cases of viral infection, we performed an epidemiologic analysis and analyzed the clinical manifestations and prognosis for the hospitalized patients.

The clinical diagnoses for acute viral LTRI cases included 58 cases of pneumonia, 25 cases of tracheobronchitis, and 4 cases of bronchiolitis. Pneumonia was most commonly caused by adenovirus (46 [79.3%] cases) (Table 1). Tracheobronchitis was most commonly caused by influenza A virus and adenovirus (14 [56.0%] and 9 [36.0%] cases, respectively). Two cases each of influenza A virus infection and parainfluenza virus infection were noted among the 4 patients with bronchiolitis. The monthly distribution of the identified viruses is shown in the online Technical Appendix Figure.

Of the 87 patients with an LTRI, 64 were hospitalized. Among these 64 patients, 49 (76.6%) had adenovirus infections, 13 (20.3%) had influenza A or B virus infections, and 2 had bronchiolitis caused by parainfluenza virus infection. Table 2 shows the clinical features of the 62 patients hospitalized for adenovirus or influenza A or B virus infection, among whom the mean age was 19.6 ± 1.1 years (adenovirus patients) and 20.1 ± 2.0 years (influenza patients). Except for a female patient infected with influenza A virus, all patients

Author affiliations: The Armed Forces Capital Hospital, Seongnam, South Korea (J.Y. Heo, J.E. Lee, K.-W. Choe); and Catholic University College of Medicine, Seoul, South Korea (H.K. Kim)

DOI: http://dx.doi.org/10.3201/eid2005.131692

[1]These authors contributed equally to this article.

Table 1. Causes of and diagnoses for acute lower respiratory tract infections in soldiers, South Korea, April 2011–March 2012

Virus identified*	Clinical diagnosis, no. (%) patients			
	Pneumonia, n = 58	Tracheobronchitis, n = 25	Bronchiolitis, n = 4	Total, N = 87
Adenovirus†	46 (79.3)	9 (36.0)	0	55 (63.2)
Influenza A	10 (17.2)	14 (56.0)	2 (50.0)	26 (29.9)
Influenza B	2 (3.5)	2 (8.0)	0	4 (4.6)
Rhinovirus group A†	2 (3.5)	1 (4.0)	0	3 (3.4)
Parainfluenza	0	0	2 (50.0)	2 (2.3)

*A total of 207 nasopharyngeal swab specimens were collected from soldiers with acute lower respiratory infections. Within 3 days of collection, the specimens were tested against 12 respiratory viruses at GClabs (Yongin-si, South Korea) by using Seeplex RV12 ACE Detection (Seegene, Seoul, South Korea).
†Three patients were co-infected with rhinovirus group A infection and adenovirus.

were male. The proportion of new military recruits was significantly higher among patients with adenovirus infection than among patients with influenza A or B virus infection (p = 0.011). There was no difference in the clinical signs and symptoms and radiographic findings of patients infected with influenza virus and adenovirus (online Technical Appendix). Mechanical ventilation was required for 6 patients in whom severe pneumonia developed; all 6 patients had adenovirus infection and 3 died (online Technical Appendix).

Conclusions

Among the viruses identified as causing acute LTRI in South Korean soldiers, adenovirus was the most common, causing 63.2% of the cases. In addition, adenovirus was identified in 79.3% of pneumonia cases. These findings are similar to those in studies among US military (*1*–*4*).

Adenovirus infection was more common among new recruits in the South Korean military. However, the infection was also confirmed in all 11 (64.7%) privates among the 17 active duty soldiers at advanced training sites (Table 2). In the South Korea military, adenovirus might have spread to secondary training sites through recruit redeployment, similar to the experience in the US military (*14*).

Cases of severe pneumonia requiring mechanical ventilation were not observed among patients with influenza

Table 2. Demographic, clinical, and laboratory findings for soldiers hospitalized with acute lower respiratory infections, South Korea, April 2011–March 2012

Variable	Soldiers hospitalized for infection with		p value*
	Adenovirus, n = 49 (79.0%)	Influenza A or B virus, n = 13 (21.0%)	
Demographic characteristics			
Age, mean y ± SD	19.63 ± 1.16	20.15 ± 2.03	0.232
Male sex	49 (100.0)	12 (92.3)	0.210
Military rank, no. (%)			0.011
New recruit	32 (65.3)	3 (23.1)	
Active-duty soldier	17 (34.7)†	10 (76.9)	
Clinical characteristics, no. (%)			
Fever ≥5 d	27 (55.1)	3 (23.1)	0.061
Cough	47 (95.9)	11 (84.6)	0.191
Rhinorrhea	29 (59.2)	7 (53.8)	0.729
Sputum	32 (65.3)	7 (53.8)	0.447
Sore throat	30 (61.2)	8 (61.5)	0.984
Dyspnea	9 (18.4)	2 (15.4)	1.000
Nausea/vomiting	8 (16.3)	3 (23.1)	0.685
Diarrhea	13 (26.5)	2 (15.4)	0.493
Chest pain	5 (10.2)	1 (7.7)	1.000
Laboratory findings ± SD			
Leukocyte count (cell/μL)	6,529 ± 2,643	8,110 ± 2,331	0.054
Hemoglobin (g/dL)	14.0 ± 0.9	13.5 ± 1.1	0.384
Platelet count (10^3cell/μL)	156 ± 29	201 ± 26	<0.001
C-reactive protein(mg/dL)	12.0 ± 3.0	8.5 ± 2.3	<0.001
Radiograph findings, no. (%)			
Consolidation	20 (40.8)	2 (15.4)	0.112
Peribronchial infiltration	26 (53.1)	8 (61.5)	0.585
Effusion	9 (18.4)	1 (7.7)	0.673
Normal	3 (6.1)	3 (23.1)	0.100
Length of hospital stay, mean d ± SD	17.1 ± 4.2	14.3 ± 4.1	0.036
Required mechanical ventilation, no. (%)	6 (12.2)	0	0.328
Died, no. (%)	3 (6.1)	0	1.000

*p<0.05 was considered significant. The statistical analyses used in this study are described in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/5/13-1692-Techapp1.pdf).
†Among the 17 hospitalized active-duty soldiers with adenovirus infection, those ranked as privates were the most common (11/17 [64.7%]). All privates who were found to have adenovirus infection had been relocated to advanced training sites after graduating from the 6-week basic military training course, which suggests that adenovirus might have spread to secondary training sites through recruit redeployment.

virus infection; however, 3 of the 6 patients with severe adenovirus-associated pneumonia died. This finding indicates that adenovirus infection is a major cause of death among soldiers with severe respiratory diseases. In 2009, in response to the influenza A(H1N1)pdm09 pandemic, multiplex reverse transcription PCR for respiratory viruses became available in South Korea military hospitals. Before 2009, apart from research purposes, clinical testing for respiratory viruses was not routinely performed at military hospitals. For this reason, South Korea military physicians were unaware that adenovirus was a major cause of respiratory disease among soldiers. Because of the lack of an effective treatment for adenovirus infection, vaccination against adenovirus has been introduced for persons, such as military recruits, at high risk for infection.

Our study has certain limitations. First, we were unable to confirm the serotype of adenovirus for positive case-patients. Because military physicians were unaware that adenovirus was a major cause of acute respiratory disease, additional serotyping was not performed at the time of diagnosis. Second, the period of study was only 1 year. The level of an epidemic varies at different times, thus requiring longer study periods to observe patterns. Third, we retrospectively reviewed medical records of patients with acute LTRI in a central referral military hospital; thus, the study patients may not be entirely representative of South Korea soldiers with acute LTRI. Last, there was a sampling bias with regard to the molecular assay; the test may have been performed only in cases of severe illness or when recommended by clinicians. Nevertheless, our results suggest that among South Korea soldiers with acute LTRIs, those with adenovirus rather than other respiratory infections had more severe clinical outcomes.

Further studies are required to determine the serotype(s) of adenovirus causing infection among the military, and epidemiologic surveillance for adenovirus is needed. In addition, studies on the effectiveness of adenovirus vaccine should be considered.

Acknowledgments

We thank staff at GClabs, Yongin-si, South Korea, for technical assistance.

This study was supported by a fund from the Armed Forces Medical Command, South Korea.

Dr Heo is a military physician in the Division of Infectious Diseases at the Armed Forces Capital Hospital. His current research interest is the epidemiology of vaccine-preventable and vector-borne diseases in military personnel in South Korea.

Dr Lee is a civilian physician in the Division of Pulmonary and Critical Care Medicine at the Armed Forces Capital Hospital. Her primary research interests are pulmonary diseases of both infectious (pneumonia and tuberculosis) and noninfectious (e.g., idiopathic acute eosinophilic pneumonia) origin that occur frequently in military personnel in South Korea.

References

1. Pazzaglia G, Pasternack M. Recent trends of pneumonia morbidity in US Naval personnel. Mil Med. 1983;148:647–51.
2. Gray GC, Goswami PR, Malasig MD, Hawksworth AW, Trump DH, Ryan MA, et al. Adult adenovirus infections: loss of orphaned vaccines precipitates military respiratory disease epidemics. Clin Infect Dis. 2000;31:663–70. http://dx.doi.org/10.1086/313999.
3. Russell KL, Hawksworth AW, Ryan MA, Strickler J, Irvine M, Hansen CJ, et al. Vaccine-preventable adenoviral respiratory illness in US military recruits, 1999–2004. Vaccine. 2006;24:2835–42. http://dx.doi.org/10.1016/j.vaccine. 2005.12.062.
4. Gray GC, Callahan JD, Hawksworth AW, Fisher CA, Gaydos JC. Respiratory diseases among US military personnel: countering emerging threats. Emerg Infect Dis. 1999;5:379–85. http://dx.doi.org/10.3201/eid0503.990308.
5. Erdman DD, Xu W, Gerber SI, Gray GC, Schnurr D, Kajon AE, et al. Molecular epidemiology of adenovirus type 7 in the United States, 1966–2000. Emerg Infect Dis. 2002;8:269–77. http://dx.doi.org/10.3201/eid0803.010190.
6. Hilleman MR. Epidemiology of adenovirus respiratory infections in military recruit populations.Ann N Y Acad Sci. 1957;67:262–72. http://dx.doi.org/10.1111/j.1749-6632.1957.tb46049.x.
7. Barraza EM, Ludwig SL, Gaydos JC, Brundage JF. Reemergence of adenovirus type 4 acute respiratory disease in military trainees: report of an outbreak during a lapse in vaccination. J Infect Dis. 1999;179:1531–3. http://dx.doi.org/10.1086/314772.
8. Blasiole DA, Metzgar D, Daum LT, Ryan MA, Wu J, Wills C, et al. Molecular analysis of adenovirus isolates from vaccinated and unvaccinated young adults. J ClinMicrobiol.2004;42:1686–93. http://dx.doi.org/10.1128/JCM.42.4.1686-1693.2004.
9. Gaydos CA, Gaydos JC. Adenovirus vaccines in the US military. Mil Med. 1995;160:300–4.
10. Hoke CH Jr, Hawksworth A, Snyder CE Jr. Initial assessment of impact of adenovirus type 4 and type 7 vaccine on febrile respiratory illness and virus transmission in military basic trainees, March 2012. MSMR. 2012;19:2–4.
11. Jeon K, Kang CI, Yoon CH, Lee DJ, Kim CH, Chung YS, et al. High isolation rate of adenovirus serotype 7 from South Korean military recruits with mild acute respiratory disease. Eur J ClinMicrobiol Infect Dis. 2007;26:481–3. http://dx.doi.org/10.1007/s10096-007-0312-6.
12. Heo JY, Kim HK, Cha YJ, Lee JE, Shim YS, Choe KW. A clinical features of severe adenovirus pneumonia among members of the Korea military: a case series [in Korean]. Infection and Chemotherapy. 2012;44:372–6.
13. Hwang SM, Park DE, Yang YI, Park SJ, Lee HK, Kim MJ, et al. Outbreak of febrile respiratory illness caused by adenovirus at a South Korean military training facility: clinical and radiological characteristics of adenovirus pneumonia. Jpn J Infect Dis. 2013;66:359–65.
14. Trei JS, Johns NM, Garner JL, Noel LB, Ortman BV, Ensz KL, et al.Spread of adenovirus to geographically dispersed military installations, May–October 2007. Emerg Infect Dis. 2010;16:769–75. http://dx.doi.org/10.3201/eid1605.091633.

Address for correspondence: Kang-Won Choe, Department of Internal Medicine, The Armed Forces Capital Hospital, 2 Gil 66 Ho Yuldong, Bundanggu, Seongnam, Kyeonggido 463-040, South Korea; email: cheokw@snu.ac.kr

Influenza-associated Hospitalizations and Deaths, Costa Rica, 2009–2012

Guiselle Guzman Saborío, Alexey Clara, Antonio Garcia, Fabio Quesada, Rakhee Palekar, Percy Minaya, Marvin Cervantes, Mariel Lopez, Jenny Lara, Jorge Jara, Natalia Blanco, Joseph Bresee, Marc-Alain-Widdowson, and Eduardo Azziz-Baumgartner

Data needed to guide influenza vaccine policies are lacking in tropical countries. We multiplied the number of severe acute respiratory infections by the proportion testing positive for influenza. There were ≈6,699 influenza hospitalizations and 803 deaths in Costa Rica during 2009–2012, supporting continuation of a national influenza vaccine program.

During 2002–2008, 41,000–160,000 persons died from influenza-associated illnesses throughout the Americas (*1*). Although it is known that influenza is preventable by use of vaccines (*2*), documentation of the value of vaccination, including data on influenza-associated hospitalizations and deaths, is limited in middle-income tropical countries.

In 2004, Costa Rica, an upper–middle income country (*3*), recommended influenza vaccines for children 6 months–8 years of age who had pre-existing conditions and for persons ≥65 years of age (*4*) although national data to support this recommendation were limited. We used the World Health Organization International Classification of Diseases, 10th Revision (ICD-10) hospital discharge criteria (*5*), the Costa Rica National Influenza Center surveillance (*6*), and census population data (*7*) to quantify influenza-associated hospitalization and mortality rates during 2009–2012 to guide national and subregional vaccine policy.

Author affiliations: Caja Costarricense del Seguro Social, San Jose, Costa Rica (G. Guzman Saborío , A. Garcia, F. Quesada, M. Cervantes); US Centers for Disease Control Central American Regional, Guatemala City, Guatemala (A. Clara); Pan American Health Organization, Washington, DC, USA (R. Palekar); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (R. Palekar, J. Bresee, M-A Widdowson, E. Azziz-Baumgartner); Training of Epidemiology and Public Health Intervention Network, Guatemala City (P. Minaya); Instituto Costarricense de Investigación y Enseñanza en Nutrición y en Salud, San Jose (M. Lopez, J. Lara); and Universidad del Valle de Guatemala, Guatemala City (J. Jara, N. Blanco)

DOI: http://dx.doi.org/10.3201/eid2005.131775

The Study

In 2005, Costa Rica maintained a database of persons hospitalized throughout the country's public hospitals. Approximately 95% of all hospitalizations occur within this centralized health care system. This hospital database contains information on patients' demographics, their first 6 ICD-10 coded diagnoses, and their survival. We used this information to determine the number of persons admitted to public hospitals during 2009–2012 and discharged with proxy diagnoses of severe acute respiratory infection (SARI) (ICD-10 code J9–18) (*8*). We used viral surveillance data to estimate the proportion of influenza-positive specimens if all had been tested by using published methods (*9*).

Influenza surveillance is conducted by Costa Rica's National Influenza Center and the *Instituto Costarricense de Investigación y Enseñanza en Nutrición y Salud* (INCIENSA). In 2002, INCIENSA laboratory staff began testing clinical nasal and pharyngeal samples for influenza by using indirect immunofluorescence. Starting in 2007, Costa Rica systematically identified 5 SARI case-patients (defined as patients with fever and cough or sore throat with respiratory difficulties requiring hospitalization) per sentinel site per week (*6*) to supplement specimens obtained through routine clinical practice. Influenza surveillance was fully operational by the 2009 pandemic year (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1775-Techapp1.pdf). INCIENSA primarily tested respiratory samples through indirect immunofluorescence and subtyped positive specimens through real-time reverse transcription PCR.

We stratified the percent of influenza-positive samples among SARI case-patients into groups of patients who were <5, 5–59, and ≥60 years of age to broaden the implications of our findings for tropical countries. For each age group, we multiplied the monthly number of SARI case-patients by the proportion of influenza-positive samples from SARI case-patients (Figure) and their 95% CI (*9*).

We assumed that all persons living in Costa Rica were at risk for development of SARI and being admitted to a public hospital. We divided the estimated annual number of influenza-associated hospitalizations and deaths by the census projections for each year and age group (*7*). We stratified our influenza analyses into pandemic (2009–2010) and seasonal influenza years (2011–2012) because we anticipated that case ascertainment, health utilization, hospital admissions, respiratory specimen collection, and rate estimates would be different during these periods. For example, during 2009, to help the World Health Organization better characterize a novel virus, surveillance

staff supplemented systematic with snowball sampling of the contacts of persons in whom diagnoses of influenza A (H1N1)pdm09 virus were laboratory confirmed (*10*). The Institutional Committee of Bioethics in Research approved this program evaluation.

During January 2009–December 2012, we identified 30,357 persons hospitalized for treatment of SARI throughout Costa Rica, of whom ≈1/3 of case-patients belonged to each age group (29% <5 years of age, 32% 5–59 years of age , and 38% ≥60 years of age). Deaths associated with SARI occurred primarily among persons ≥60 years of age. Although 1% of children <5 years of age died during hospitalization, 12% of persons 5–59 years of age and 38% of persons ≥60 years of age died while hospitalized (p<0.001).

A total of 16,582 of SARI case-patients were tested for influenza. Of these, 8,158 (49%) were female. More persons were tested during the 2009–2010 pandemic (9,879) than during 2011–2012 (6,703). Of the 16,597 case-patients tested, 3,352 (20%) tested positive for influenza (Table).

We estimated that 6,699 (95% CI 5,165–8,264) influenza hospitalizations occurred among all age groups (Table). These represented 0.4 hospitalizations for influenza per 1,000 person-years (py) (95% CI 0.3–0.4/1,000py). The rate of influenza-associated hospitalizations was higher during the 2009–2010 pandemic (mean 0.6/1000 py) compared with those during 2011– 2012 (mean 0.1/1000 py) (p<0.001) (Figure).

We estimated that 803 (95% CI 474–1,149) in-hospital deaths attributed to influenza occurred among all age groups (Table); 628 (78%) occurred during the 2009-2010 pandemic years. Influenza-associated deaths represented 12% of influenza-associated SARI hospitalizations and a rate of 4/100,000 py. Deaths occurred primarily among persons ≥60 years of age, for whom the influenza mortality rate was 29/100,000 py compared with 2/100,000 py

Table. Influenza-associated SARI hospitalizations and deaths in Costa Rica, 2009–2012*

Year and age group, y	Population size	No. hospitalizations for SARI†	No. deaths‡	No. (%) influenza-positive respiratory samples§	Influenza-associated hospitalizations		Influenza-associated deaths	
					No. (95% CI)¶	Rate# (95% CI)¶	No. (95% CI)¶	Rate** (95% CI)¶
2009								
<5	356,266	2,196	17	194/1,349 (14)	436 (336–535)	1.2 (0.9–1.5)	2 (1–3)	0.6 (0.3–0.8)
5–59	3,700,000	2,998	328	1,608/3,653 (44)	1,852 (1,737–1,977)	0.5 (0.47–0.53)	115 (95–138)	3.1 (2.6–3.7)
≥60	415,210	2,797	1,042	265/1,306 (20)	657 (513–804)	1.6 (1.2–1.9)	171 (112–231)	41 (27–56)
2010								
<5	350,902	2,583	27	185/1,230 (15)	529 (366–688)	1.5 (1.0–2.0)	4 (2–4)	1.1 (0.6–1.1)
5–59	3,800,000	2,595	317	507/1,472 (34)	1,185 (993–1,378)	0.3 (0.3–0.4)	107 (78–133)	2.8 (2.1–3.5)
≥60	444,981	3,073	1,172	161/879 (18)	742 (467–1,019)	1.7 (1.0–2.3)	229 (123–340)	51 (28–76)
2011								
<5	347,888	2,204	32	48/1,797 (3)	114 (67–161)	0.3 (0.2–0.5)	0	0
5–59	3,800,000	2,224	330	40/700 (6)	174 (72–286)	0.05 (0.02–0.08)	20 (6–38)	0.5 (0.2–1.0)
≥60	463,100	3,049	1,183	27/639 (4)	163 (53–279)	0.4 (0.1–0.6)	52 (12–96)	11 (3–21)
2012								
<5	344,577	1,927	22	79/1,526 (5)	164 (109–216)	0.5 (0.3–0.6)	1 (0–2)	0.3 (0.0–0.6)
5–59	3,900,000	2,016	229	162/1,034 (16)	423 (307–540)	0.1 (0.08–0.14)	31 (18–46)	0.8 (0.5–1.2)
≥60	481,557	2,695	1,051	76/1,012 (8)	260 (145–381)	0.5 (0.3–0.8)	71 (27–118)	15 (6–25)
Total	18,404,481	30,357	5,750	3,352/16,597 (20)	6,699 (5,165–8,264)	0.4 (0.3–0.4)	803 (474–1,149)	4 (3–6)

*SARI, severe acute respiratory illness. Population estimates based on Costa Rica census projections.
†Number of persons hospitalized during 2009–2012 for SARI proxy diagnoses (ICD-10 code J9–18) (*8*) in any of 6 discharge diagnostic fields.
‡In-hospital deaths among persons hospitalized during 2009–2012 for SARI (International Classification of Diseases, 10th Revision, code J9–18) in any of 6 discharge diagnostic fields.
§Annual number of nasal and pharyngeal specimens positive for influenza by immunofluorescence and PCR over total number tested.
¶Estimated through the product of the proportion of samples testing positive for influenza and the number of persons hospitalized for or inpatient deaths from SARI each month and sum of the products by age group, Costarricense de Seguro Social, San José, Costa Rica.
#Per 1,000 person-years.
**Per 100,000 person-years.

Figure. Hospitalizations for severe acute respiratory infection and proportion of samples testing positive for influenza in Costa Rica during 2009–2012. National Influenza Centre and influenza sentinel surveillance sites in the provinces of San José, Costa Rica.

among other age groups (p<0.001). During the 2009–2010 pandemic, 47 persons ≥60 years of age per 100,000 py died as a result of influenza-associated SARI, compared with 3 persons <60 years of age per 100,000 py.

Conclusions

Our study suggests that Costa Rica had substantive numbers of influenza-associated hospitalizations and deaths. Hospitalization rates were highest among children <5 years of age and persons ≥60 of age. Although deaths increased during the pandemic among persons 5–59 years of age, persons ≥60 years of age were more likely to die as a result of influenza. Our findings support the decision of the officials of Costa Rican Social Security Board to provide seasonal influenza vaccination for children and older adults, and for groups that may be different during pandemics.

The influenza-associated hospitalization and mortality rates in our study were within the range of those estimated in middle-income countries in the American tropics. Pandemic influenza-associated hospitalization rates among children in Costa Rica were within the range of the influenza-associated severe pneumonia hospitalization rates in El Salvador among children <5 years of age (*9*), but higher than rates estimated from population-based surveillance in Guatemala (0.3/1000 py) (*11*). The rates calculated for this study were lower than those estimated for deaths from pneumonia associated with influenza in Brazil (1.4, 95% CI 0.7–2.1 /100,000 py) (*12*). Our estimated influenza-associated mortality rates were similar, however, to deaths attributed to influenza-associated respiratory illness in Mexico (3.7 [95% CI 3.0–4.4]/100,000 population) (*13*). Rates were generated by using different case definitions, surveillance, and analytical methods. Standardization of surveillance (*6*) and the methods to estimate burden of disease may yield estimates more readily compared among countries (*8*).

This study had several limitations. We were unable to directly measure rates of laboratory-confirmed influenza among all hospitalized case-patients. Respiratory samples were from clinical specimens gathered during routine practice and from systematically identified patients. A different proportion of persons discharged from the hospital or who died after receiving a diagnosis of SARI may have tested positive for influenza than the proportion tested by convenience. Persons with respiratory illness may have been more likely to be admitted during the pandemic, thus inflating hospitalization rates during 2009–2010 when compared with 2011–2012. Also, a larger but unquantified proportion of samples were first tested through indirect immunofluorescence rather than the more sensitive PCR during 2011–2012 than during the pandemic (*14*).

Our study suggests that Costa Rica had substantive influenza-associated hospitalization and mortality rates, particularly among the very young and the elderly. Influenza-associated hospitalization and mortality rates were similar to those of neighboring countries that routinely dispense influenza vaccines and oseltamivir. Further studies may be warranted to explore the value and sustainability of expanded influenza vaccination programs among populations most at risk for development of severe illness.

Acknowledgments

We acknowledge the work of the staff of the Sentinel Site Epidemiologic Surveillance Unit at the South Central Health Region and the Max Peralta sentinel hospital, the leadership at Caja Costarricense de Seguro Social (Medical Management, Health Services Development Unit, South Central Health Services Network Unit), Instituto Nacional de Estadística y Censo, INCIENSA, Fundación del Centro Nacional de Alta Tecnología (Fun-CENAT) and PRIAS-CENAT that generated the data and supported for this study. We also acknowledge the leadership of the Centers for Disease Control and Prevention, Influenza

Division and the Pan American Health Organization, who facilitated improvements in influenza surveillance and influenza vaccine policies throughout the Americas and underscored the need to generate influenza burden estimates in order to better inform influenza vaccine policy.

This article is dedicated to the memory of Alexander Klimov, friend and mentor (March 25, 1943–February 5, 2013).

This investigation has been funded in part by the Centers for Disease Control and Prevention.

Dr Guzmán Saborío is chief of Collective Health and Influenza Surveillance at Caja Costarricense de Seguro Social. Her research interests include infectious disease surveillance, public health emergency preparedness, and influenza and other respiratory viruses.

References

1. Cheng PY, Palekar R, Azziz-Baumgartner E, Marinho F, Iuliano D, Glew P, et al. Regional estimates of influenza mortality. Achievements and future challenges in the surveillance of respiratory viruses; 2013 January 29–30, 2013; San Jose (Costa Rica): US Centers for Disease Control and Prevention; 2013.
2. Centers for Disease Control and Prevention. CDC Says "Take 3" Actions to fight the flu. 2010 [cited 2013 Nov 1]. http://www.cdc.gov/flu/protect/preventing.htm
3. World_Bank. How we Classify Countries. 2010 [cited 2010 Dec 5]. http://data.worldbank.org/about/country-classifications
4. Ropero-Álvarez A, Kurtis HJ, Danovaro-Holliday M, Ruiz-Matus C, Andrus J. Expansion of seasonal influenza vaccination in the Americas. BMC Public Health. 2009;9:361–9. http://dx.doi.org/10.1186/1471-2458-9-361
5. World Health Organization. International Classification of Diseases (ICD) 2011 [cited 2012 Sep 2]. http://www.who.int/classifications/icd/en/
6. Pan American Health Organization-Centers for Disease Control and Prevention. Generic Protocol for Influenza Surveillance 2006 [cited 2012 Jul 15]. http://www1.paho.org/English/AD/DPC/CD/flu-snl-gpis.pdf
7. Instituto Nacional de Estadística y Censo CR. Poblaciones. 2000 [cited 2013 Nov 1]. http://www.inec.go.cr/Web/Home/GeneradorPagina.aspx
8. Nair H, Campbell H, Mounts A. A manual for estimating disease burden associated with seasonal influenza in a population. Geneva: World Health Organization; 2009.
9. Clara W, Armero J, Rodriguez D, Lozano C, Bonilla L, Minaya P, et al. Estimated incidence of influenza-associated severe pneumonia rates in children younger than 5 years in El Salvador, 2008–2010. Bull World Health Organ. 2012; 90:756–63. PubMed http://dx.doi.org/10.2471/BLT.11.098202
10. Writing Committee of the WHO. Clinical aspects of pandemic (H1N1) 2009 influenza. N Engl J Med. 2010;362:1708–19. http://dx.doi.org/10.1056/NEJMra1000449
11. Reyes L, Arvelo W, Estevez A, Gray J, Moir J, Gordillo B, et al. Population-based surveillance for 2009 pandemic influenza A (H1N1) virus in Guatemala, 2009. Influenza Other Respi Viruses. 2010;4:129–40. http://dx.doi.org/10.1111/j.1750-2659.2010.00138.x
12. Freitas FT, Souza LR, Azziz-Baumgartner E, Cheng PY, Zhou H, Widdowson MA, et al. Influenza-associated excess mortality in southern Brazil, 1980–2008. Epidemiol Infect. 2013;141:1731–40. PubMed http://dx.doi.org/10.1017/S0950268812002221
13. Charu V, Chowell G, Mejia L, Echevarria-Zuno S, Borja-Aburto V, Simonsen L, et al. Mortality burden of the A/H1N1 pandemic in Mexico: a comparison of deaths and years of life lost to seasonal influenza. Clin Infect Dis. 2011;53:985–93. http://dx.doi.org/10.1093/cid/cir644
14. Atmar R, Baxter B, Dominguez E, Taber L. Comparison of reverse transcription-PCR with tissue culture and other rapid diagnostic assays for detection of type A influenza virus. J Clin Microbiol. 1996;34:2604–6.

Address for correspondence: Guiselle Guzmán, Caja Costarricense de Seguro Social, San José, Costa Rica, Av Segunda Oficinas Centrales CCSS, Edificio Genaron Valverde, Piso 13, Área de Salud Colectiva, San Jose, Costa Rica; email: gguzmans@ccss.sa.cr

Responses to Threat of Influenza A(H7N9) and Support for Live Poultry Markets, Hong Kong, 2013

Peng Wu, Vicky J. Fang, Qiuyan Liao, Diane M.W. Ng, Joseph T. Wu, Gabriel M. Leung, Richard Fielding, and Benjamin J. Cowling

We conducted a population survey in Hong Kong to gauge psychological and behavioral responses to the threat of influenza A(H7N9) and support for closure of live poultry markets. We found low anxiety and low levels of exposure to live poultry but mixed support for permanent closure of the markets.

A novel influenza A(H7N9) virus was detected in China in March 2013, and an epidemic of infections in poultry and humans occurred during April and May of that year (*1*,*2*). Most persons who had laboratory-confirmed infections reported recent contact with live poultry, and evidence suggests that human-to-human transmissibility of the virus is low (*2*). Incidence of laboratory-confirmed human cases dramatically decreased following the closure of live poultry markets (LPMs) in affected cities in April 2013 (*3*). Control of the virus is challenging because its low pathogenicity in poultry (*4*) requires reliance on laboratory-based surveillance in animals or humans to identify areas where the virus is prevalent and to facilitate interventions to reduce human exposure to infected poultry.

Hong Kong imports live poultry from mainland China only from a few dedicated farms that have adequate biosecurity protection; intensive surveillance for avian influenza is conducted at the border and within Hong Kong. Risk for influenza A(H7N9) virus infection appears to be low in Hong Kong, but 4 infections have been reported in Hong Kong residents since December 2013, and a surge in influenza virus transmission was anticipated in eastern China this winter (*2*). Prevention and control activities rely on accurate measures of exposure to live poultry, risk perception and psychological and behavioral responses related to the virus, and attitudes toward specific control measures. We therefore conducted a series of cross-sectional population surveys to monitor these variables in Hong Kong.

Author affiliation: School of Public Health, The University of Hong Kong, Hong Kong, China

DOI: http://dx.doi.org/10.3201/eid2005.131859

The Study

We initiated the first survey in April 2013 (April 10–13 and 25–27), shortly after the first human case of influenza A(H7N9) was announced in mainland China. A second survey was conducted December 4–8, after incidence of human cases began to rise in the winter and the first local infection occurred in Hong Kong. We used methods and survey instruments similar to those used for surveys during the severe acute respiratory syndrome epidemic in 2003 (*5*), the influenza A(H1N1)pdm09 pandemic (*6*), and the emergence of avian influenza A(H5N1) (*7*).

For the survey, trained interviewers made telephone calls to land lines by using a computerized, random-digit dialing system; calls were placed during nonworking hours and weekends to avoid overrepresentation of nonworking groups. Within households, adults ≥18 years of age who spoke Cantonese were eligible and were randomly selected on the basis of a Kish grid (*8*). Up to 4 follow-up calls were made if participants were not available or if calls were unanswered. Verbal informed consent was obtained from all participants. Means and proportions of survey items were directly weighted by sex and age to the general population, and multiple imputation with 10 datasets was used to correct for missing data.

We completed 1,556 interviews during the April survey and 1,000 interviews during the December survey; response rates were 68.9% and 68.0%, respectively. The characteristics of respondents were similar for each survey period (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/5/13-1859-Techapp1.pdf). Figure 1 illustrates the timeline of laboratory-confirmed cases of influenza A(H7N9) compared with the dates of our surveys.

Table 1 shows a summary of the population anxiety and risk perception related to influenza A(H7N9) across the 2 survey periods. The level of general anxiety in the population, measured by the State Trait Anxiety Inventory (*5*,*6*), remained low and was comparable to population anxiety during April–May 2009 (*6*). Most (85%–89%) respondents identified close contact with chickens in an LPM as a risk factor for infection with influenza A(H7N9) virus. Respondents reported perceived susceptibility of infection as low, perceived severity of influenza A(H7N9) as lower than that of severe acute respiratory syndrome, and perceived severity of influenza A(H7N9) as higher than that of influenza A(H5N1) and seasonal influenza. Respondents also expressed that they would experience low levels of worry if influenza-like symptoms were to develop in the respondent the day after the survey (symptom-induced worry).

During the December survey, we also collected data on exposures to live poultry markets (LPMs). A total of 26.7%

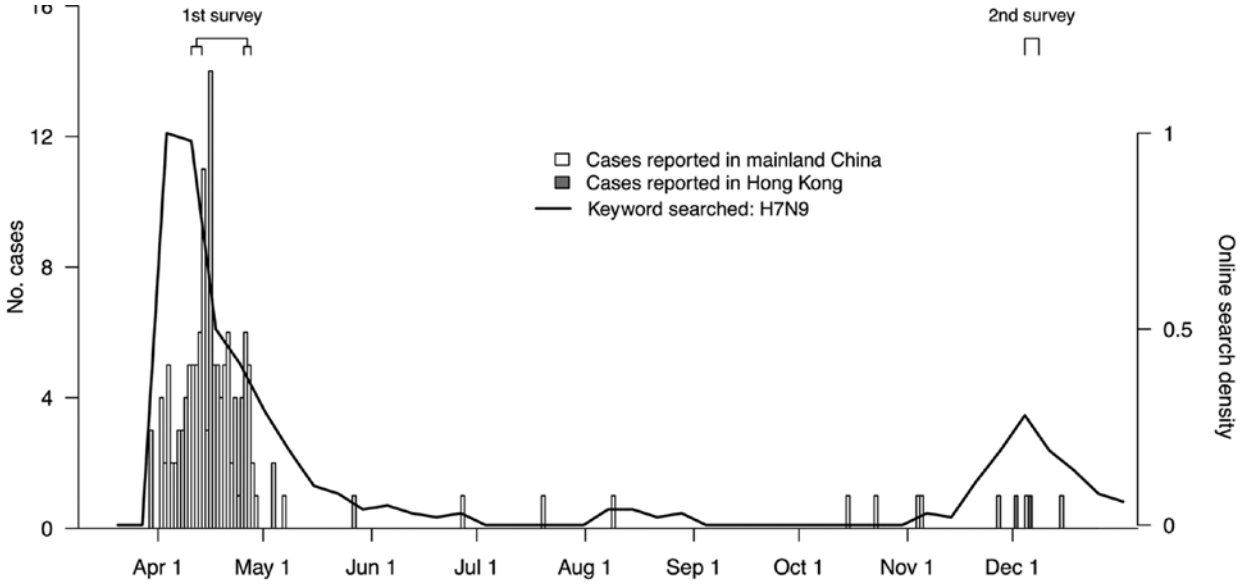


Figure 1. Laboratory-confirmed human cases of influenza A(H7N9) virus infection in mainland China and Hong Kong, by date of announcement, compared with timing of population surveys and public interest in influenza A(H7N9), 2013. Public interest was calculated by using Google Trends (www.google.com/trends) on the basis of internet searches on the keyword H7N9 measured by normalized relative search volume; lines show the ratio of weekly search volume on the defined keywords divided by the search volume on any keyword during the period, after normalizing the highest ratio as 1. April survey was conducted in 2 phases; information was combined for these analyses.

of respondents reported visiting an LPM in Hong Kong ≥1 time during the previous year; of those, 61.4% and 37.5% reported visiting ≥1 time per month and ≥1 time per week, respectively. In addition, 6.9% of respondents reported visiting an LPM in mainland China ≥1 time during the previous year. Across the population, we estimated the average numbers of annual visits to LPMs in Hong Kong and mainland China to be 17.6 and 0.53 visits per person, respectively. These estimates were based on an assumption of standardized annual numbers of visits for responses 1–2/year, 3–5/year, 6–11/year, 1–3/month, 1–2/week, 3–5/week, almost every day, or never to be 1.5, 4, 8.5, 24, 78, 208, 365, and 0 visits, respectively. Figure 2 shows the distribution of number of visits by age and sex; in a multiple regression model, we found significantly ($p<0.01$) fewer visits to LPMs among younger adults but no significant differences by sex.

A total of 17.5% respondents reported that they had avoided visiting LPMs in the previous 7 days because of influenza A(H7N9), whereas 35.9% reported that they would support or strongly support permanent closure of LPMs.

Table 1. Generalized anxiety and risk perception among persons surveyed during influenza A(H7N9) epidemic, Hong Kong, April and December 2013*

Category	First survey	Second survey	p value
Population anxiety†	1.81	1.79	0.35
Risk perception, %			
Susceptibility‡			
Absolute	12.1	9.3	0.01
Relative	2.3	1.3	0.19
Severity compared with seasonal influenza§	88.1	88.3	0.80
Severity compared with SARS§	39.5	28.8	<0.01
Severity compared with influenza A(H5N1)§	79.1	81.6	0.10
Symptom-induced worry¶	44.8	37.3	<0.01

*First survey conducted April 10–13 and 25–27, 2013; second survey conducted December 4–8, 2013. p values were estimated by comparing anxiety and risk perception between the 2 surveys after adjustment for demographics including age, sex, education, place of birth, marital status, and household income. SARS, severe acute respiratory syndrome.
†Measured by 4-point State Trait Anxiety Inventory (*5*; 1 indicates least anxiety, 4 most anxiety).
‡Absolute susceptibility was examined by asking how likely the survey participant thought it was that he or she would contract influenza A(H7N9) during the next month; relative susceptibility was examined by asking how likely the survey participant thought it was that he or she would contract influenza A(H7N9) during the next month compared with persons outside his or her family with similar age. Answers were given on a 7-point scale and measured as proportion of respondents whose answer was likely, very likely, or certain.
§Perceived severity was examined by asking respondents how the severity of influenza A(H7N9) compared with that of seasonal influenza, SARS, and influenza A(H5N1). Answers were given on a 5-point scale and measured as proportion of respondents whose answer was either a bit higher or much higher.
¶Perceived anxiety level if respondent were to experience onset of influenza-like symptoms in the next day. Answers were given a 7-point scale and measured as proportion of respondents whose answer was worried more than normal, worried much more than normal, or extremely worried.

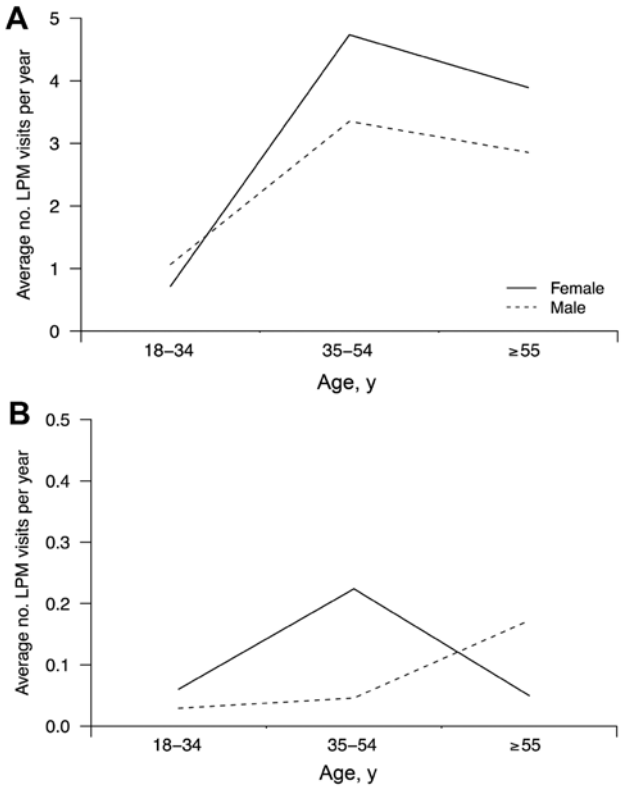


Figure 2. Exposures to live poultry among Hong Kong residents, by age and sex, in terms of weighted average numbers of visits per year to live poultry markets in Hong Kong (A) and mainland China (B).

We used multivariable logistic regression to examine factors associated with avoiding LPMs or supporting closure of LPMs (Table 2) and found that more symptom-induced worry and higher perceived severity compared with seasonal influenza were associated with avoiding visiting LPMs. Younger age, lower educational attainment, and having visited LPMs ≥1 time in the preceding year were independently associated with a lower probability of support for permanent LPM closures.

Conclusions

Results from previous studies and our surveys indicate that exposure to poultry measured by LPM visits among the Hong Kong population has declined since 2006 (*7*) and is lower than that for cities in southern China (*9*–*11*). Contact with live poultry or visiting LPMs was reported by most persons with confirmed influenza A(H7N9) in China (*2*). Four recently reported influenza A(H7N9) cases in Hong Kong were suspected to be imported; all 4 patients reported travel to Shenzhen, the city bordering Hong Kong, which reported 17 new cases during January 1–February 9, 2014. One of the patients in Hong Kong bought live poultry from an LPM in Shenzhen, where 2 LPMs subsequently yielded environmental specimens testing positive for influenza A(H7N9) virus (*12*).

We previously reported that LPM closure substantially reduced the risk for human infection with influenza A(H7N9) virus in mainland China (*3*). Control measures in LPMs in Hong Kong have become increasingly stringent during the past decade, and the current policy banning any overnight stay of live poultry in LPMs has substantially decreased avian influenza virus prevalence among poultry (*13*). This policy might also contribute to the low perceived risk for infection and low levels of symptom-induced worry observed in this study (Table 1).

Our study has limitations. Because participants were recruited on the basis of randomly selected telephone numbers, respondents might not represent the general population in Hong Kong, despite weighting of the sample by age and sex. Responses in the survey were self-reported and might be subject to response biases, including social desirability bias. We also used contact history in the previous year to measure respondents' live poultry exposure, which could be subject to recall bias.

In conclusion, our survey found generally low anxiety levels among the population in Hong Kong related to the threat of influenza A(H7N9). A higher level of symptom-induced worry and higher perceived severity of influenza A(H7N9) compared with seasonal influenza were associated with avoidance of LPMs. Permanent closure of LPMs is being considered in Hong Kong, but our results suggest that obtaining support from the public might be difficult, particularly among younger adults and adults with lower educational attainment.

This work was supported by the Area of Excellence Scheme of the Hong Kong University Grants Committee (grant no. AoE/M-12/06) and the Harvard Center for Communicable Disease Dynamics from the National Institute of General Medical Sciences (grant no. U54 GM088558). B.J.C. receives funding from MedImmune Inc. and Sanofi Pasteur and consults for Crucell NV.

Dr Wu is a postdoctoral fellow in infectious disease epidemiology at the University of Hong Kong. Her research interests include transmission dynamics of respiratory infections and the effectiveness of control measures.

References

1. Li Q, Zhou L, Zhou M, Chen Z, Li F, Wu H, et al. Epidemiology of human infections with avian influenza A(H7N9) virus in China. N Engl J Med. 2014;370:520–32. http://dx.doi.org/10.1056/NEJMoa1304617
2. Cowling BJ, Jin L, Lau EH, Liao Q, Wu P, Jiang H, et al. Comparative epidemiology of human infections with avian influenza A H7N9 and H5N1 viruses in China: a population-based study of laboratory-confirmed cases. Lancet. 2013;382:129–37. http://dx.doi.org/10.1016/S0140-6736(13)61171-X

Table 2. Factors affecting behavioral response to influenza A(H7N9) and support for permanent closure of LPMs in Hong Kong, 2013*

Characteristic	Odds ratio (95% CI)	
	Avoided visiting LPMs in previous 7 d because of influenza A(H7N9)	Would support permanent closure of LPMs
Sex		
M	Reference	Reference
F	1.07 (0.75–1.53)	1.22 (0.92–1.63)
Age, y		
18–34	Reference	Reference
35–54	0.89 (0.55–1.46)	2.28 (1.53–3.40)
≥55	0.81 (0.48–1.36)	2.87 (1.86–4.45)
Educational attainment		
Primary or below	Reference	Reference
Secondary	1.63 (0.92–2.89)	1.76 (1.17–2.65)
University or above	1.65 (0.88–3.09)	2.99 (1.88–4.76)
Visited LPM >1 time in previous year		
No	Reference	Reference
Yes	1.15 (0.78–1.68)	0.60 (0.44–0.83)
Median State Trait Anxiety score (*5*)		
<1.7	Reference	Reference
≥1.7	1.03 (0.72–1.46)	0.95 (0.72–1.25)
Self-perceived risk for infection with influenza A(H7N9)†		
Low	0.68 (0.42–1.11)	0.95 (0.64–1.42)
Evens	Reference	Reference
High	0.95 (0.49–1.84)	1.51 (0.87–2.63)
Self-perceived risk for infection with influenza A(H7N9) compared with other persons‡		
Low	1.22 (0.74–1.99)	0.93 (0.62–1.38)
Evens	Reference	Reference
High	1.97 (0.51–7.61)	0.52 (0.13–1.98)
Symptom-induced worry§		
Less	1.01 (0.62–1.64)	0.87 (0.61–1.24)
As usual	Reference	Reference
More	2.00 (1.34–2.98)	1.08 (0.78–1.48)
Perceived severity compared with seasonal influenza¶		
Less	1.22 (0.53–2.79)	1.01 (0.59–1.73)
Same	Reference	Reference
More	2.31 (1.33–3.99)	1.28 (0.87–1.87)
Perceived severity compared with SARS¶		
Less	0.84 (0.52–1.36)	0.89 (0.61–1.29)
Same	Reference	Reference
More	0.91 (0.48–1.73)	0.88 (0.52–1.48)
Perceived severity compared with influenza A(H5N1)¶		
Less	1.29 (0.73–2.26)	0.76 (0.49–1.17)
Same	Reference	Reference
More	1.06 (0.70–1.60)	0.93 (0.66–1.31)

*Odds ratios adjusted for all variables shown. LPMs, live poultry markets; SARS, severe acute respiratory syndrome.
†Respondents were asked, "How likely do you think it is that you will contract H7N9 avian flu over the next 1 month?" Low indicates the answers Never/Very unlikely/Unlikely; evens, the answer Same (50% probability); and high, the answers Likely/Very likely/Certain.
‡Respondents were asked, "What do you think are your chances of getting H7N9 avian flu over the next 1 month compared to other people outside your family of a similar age?" Low indicates the answers Not at all/Much less/Less; evens, the answer Same (50% probability); and high, the answers Likely/Very likely/Certain or More/Much more/Certain.
§Respondents were asked, "If you were to develop flu-like symptoms tomorrow, would you be…" followed by several choices. Low indicates the answers Not worried at all/Much less worried than normal/Worried less than normal; as usual, the answer About same; and more, the answers Worried more than normal/Worried much more than normal/Extremely worried.
¶Respondents were asked, "How is the severity of infection with H7N9 avian influenza compared to seasonal influenza, SARS, or H5N1 avian influenza?" Lower indicates the answers A little lower/Much lower; same, the answer Same; and higher, the answers Much higher/A little higher.

3. Yu H, Wu JT, Cowling BJ, Liao Q, Fang VJ, Zhou S, et al. Effect of closure of live poultry markets on poultry-to-person transmission of avian influenza A H7N9 virus: an ecological study. Lancet. 2014;383:541–8. http://dx.doi.org/10.1016/S0140-6736(13)61904-2
4. Uyeki TM, Cox NJ. Global concerns regarding novel influenza A (H7N9) virus infections. N Engl J Med. 2013;368:1862–4. http://dx.doi.org/10.1056/NEJMp1304661
5. Leung GM, Ho L-M, Chan SK, Ho S-Y, Bacon-Shone J, Choy RY, et al. Longitudinal assessment of community psychobehavioral responses during and after the 2003 outbreak of severe acute respiratory syndrome in Hong Kong. Clin Infect Dis. 2005;40:1713–20. http://dx.doi.org/10.1086/429923
6. Cowling BJ, Ng DM, Ip DK, Liao Q, Lam WW, Wu JT, et al. Community psychological and behavioral responses through the first wave of the 2009 influenza A (H1N1) pandemic in Hong Kong. J Infect Dis. 2010;202:867–76. http://dx.doi.org/10.1086/655811
7. Liao Q, Cowling BJ, Lam WT, Fielding R. Changing perception of avian influenza risk, Hong Kong, 2006–2010. Emerg Infect Dis. 2011;17:2379–80. http://dx.doi.org/10.3201/eid1712.110298
8. Kish L. A procedure for objective respondent selection within the household. J Am Stat Assoc. 1949;44:380–7. http://dx.doi.org/10.1080/01621459.1949.10483314
9. Cowling BJ, Freeman G, Wong JY, Wu P, Liao Q, Lau EH, et al. Preliminary inferences on the age-specific seriousness of human

disease caused by avian influenza A(H7N9) infections in China, March to April 2013. Euro Surveill. 2013;18:20475.
10. Liao Q, Lam WT, Leung GM, Jiang C, Fielding R. Live poultry exposure, Guangzhou, China, 2006. Epidemics. 2009;1:207–12. http://dx.doi.org/10.1016/j.epidem.2009.09.002
11. Liao Q, Lam WW, Jiang CQ, Ho EY, Liu YM, Zhang WS, et al. Avian influenza risk perception and live poultry purchase in Guangzhou, China, 2006. Risk Anal. 2009;29:416–24. http://dx.doi.org/10.1111/j.1539-6924.2008.01157.x
12. Centre for Health Protection, Department of Health of the Government of Hong Kong. CHP's response to media enquiries on notification from Guangdong HFPC. 2013 Dec 11 [cited 2013 Dec 16]. http://www.chp.gov.hk/en/content/116/32546.html
13. Leung YH, Lau EH, Zhang LJ, Guan Y, Cowling BJ, Peiris JS. Avian influenza and ban on overnight poultry storage in live poultry markets, Hong Kong. Emerg Infect Dis. 2012;18:1339–41. http://dx.doi.org/10.3201/eid1808.111879

Address for correspondence: Benjamin J. Cowling, School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong; email: bcowling@hku.hk

Full-Genome Analysis of Avian Influenza A(H5N1) Virus from a Human, North America, 2013

Kanti Pabbaraju, Raymond Tellier, Sallene Wong, Yan Li, Nathalie Bastien, Julian W. Tang, Steven J. Drews, Yunho Jang, C. Todd Davis, Kevin Fonseca, and Graham A. Tipples

Full-genome analysis was conducted on the first isolate of a highly pathogenic avian influenza A(H5N1) virus from a human in North America. The virus has a hemagglutinin gene of clade 2.3.2.1c and is a reassortant with an H9N2 subtype lineage polymerase basic 2 gene. No mutations conferring resistance to adamantanes or neuraminidase inhibitors were found.

Since the 1997 emergence of highly pathogenic avian influenza (HPAI) A(H5N1) virus in Hong Kong, China, 648 HPAI A(H5N1) infections and 384 associated deaths in humans have been reported. During 2013, Cambodia reported the most human infections, followed by Egypt, Indonesia, China, and Vietnam (www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/, December 10, 2013, report). In December 2013, an HPAI A(H5N1) infection was reported in a Canadian resident who recently returned from China. No human or poultry HPAI A(H5N1) infections had been previously reported in North America.

Case Report and Laboratory Investigations

Preliminary details of this case have been reported (*1*) (online Technical Appendix 1, wwwnc.cdc.gov/EID/article/20/5/14-0164-Techapp1.pdf). The patient initially sought care for respiratory symptoms; however, the probable cause of death was listed as meningoencephalitis, an unusual outcome for HPAI A(H5N1) infections in humans. Detailed interviews with close contacts have not identified exposure to infected avian sources or environmental contamination, although these investigations are continuing. Because symptom onset occurred during a return flight from China, it is probable that the patient was exposed to the virus while in China.

Nasopharyngeal swab (NP) samples, bronchoalveolar lavage (BAL), and cerebrospinal fluid (CSF) samples tested positive for influenza A(H5N1) virus by various molecular testing methods, including sequencing, at the Provincial Laboratory for Public Health and the National Microbiology Laboratory, Public Health Agency of Canada (*1*). An isolate cultured from BAL (A/Alberta/01/2014) underwent full-genome sequencing (methods available in online Technical Appendix 1); analysis results are presented here.

Partial sequences of virus from the primary specimens (shown in parentheses) included 1,378 bp of the hemagglutinin (HA) gene (CSF, BAL, NP), 1,350 bp of the neuraminidase gene (BAL), 810 bp of the matrix gene (NP), and 687 bp of the polymerase basic 2 (PB2) gene (NP). These sequences were identical to corresponding sequences obtained from the isolate, suggesting the absence of cell culture–induced changes.

BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of each gene of A/Alberta/01/2014 showed that 7 of 8 genes shared ≥99% identity at the nucleotide and protein levels with HPAI A(H5N1) viruses of avian origin. However, the PB2 gene showed 98% nt similarity and 99% aa identity to avian influenza A(H9N2) viruses collected in China. Phylogenetic analysis of each gene (online Technical Appendix 2, wwwnc.cdc.gov/EID/article/20/5/14-0164-Techapp2.pdf) with sequences from related viruses confirmed that only the PB2 gene resulted from reassortment with an avian influenza A virus containing an H9N2 subtype lineage PB2 gene (Figure 1). Phylogenetic analysis of the HA gene demonstrated that the virus belongs to clade 2.3.2.1c (*2*) (Figure 2), which has been detected in many countries and has recently been reported in China, Vietnam, and Indonesia (*2*). The HA gene of A/Alberta/01/2014 (H5N1) was most closely related to the sequence of an HPAI A(H5N1) virus from a tiger that died in 2013 at a zoo in Jiangsu, China. This combination of clade 2.3.2.1c lineage HA, neuraminidase, and internal gene segments derived from influenza A(H5N1) viruses and an H9N2 subtype lineage PB2 gene indicated that this virus is a previously undescribed genotype of HPAI A(H5N1).

To assess the virus for molecular markers of pandemic risk, we reviewed all protein sequences for mutations listed in the H5N1 Genetic Changes Inventory (*3*).The HA protein possessed a multibasic amino acid cleavage site motif (PQRERRRKR*G) similar to other clade 2.3.2.1 viruses

Author affiliations: Provincial Laboratory for Public Health, Calgary, Alberta, Canada (K. Pabbaraju, R. Tellier, S. Wong, J.W. Tang, S.J. Drews, K. Fonseca, G.A. Tipples); Public Health Agency of Canada, Winnipeg, Manitoba, Canada (Y. Li, N. Bastien); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (Y. Jang, C.T. Davis); University of Calgary, Calgary (R. Tellier, K. Fonseca, S.J. Drews); University of Manitoba, Winnipeg (Y. Li); and University of Alberta, Edmonton, Alberta, Canada (J.W. Tang, G.A. Tipples)

DOI: http://dx.doi.org/10.3201/eid2005.140164

Figure 1. Neighbor-joining phylogenetic tree of the polymerase basic 2 (PB2) genes of H9N2 subtype lineage avian influenza A viruses with A/Alberta/01/2014 (GISAID accession no. EPI500778). The avian influenza A(H5N1) virus detected in Canada is underlined. Major lineages of the H9N2 subtype–like PB2 genes are depicted to the right of the phylogenetic clusters. Bootstraps generated from 1,000 replicates are shown at branch nodes. Scale bar represents nucleotide substitutions per site. GSAID, Global Initiative on Sharing Avian Influenza Data.

(*4*). The sequence of the 220-loop receptor binding site (RBS) contained the typical avian amino acids, Q222/G224, predictive of a preference for the avian α2,3 rather than the human α2,6 sialic acid (SA) host cell receptor (*5*); all HA gene numbering is based on H5 viruses unless otherwise indicated. The RBS sequence was identical in the NP and BAL samples, suggesting the absence of adaptive changes in the cultured isolate. The G221R substitution, uncommon in HPAI A(H5N1) virus, was detected in the RBS. Previously reported in a clade 2 HPAI A(H5N1) virus (GenBank accession no. ABR13964), R221 has been shown in influenza A/H3 (R225 by H3 numbering) to slightly increase binding to human erythrocytes (*6*). Other mutations of interest in A/Alberta/01/2014 were D94N, S133A, S155N, and T156A. D94N decreased binding to α2,3 SA and increased it to α2,6 SA in a pseudotyping assay (*7*). S133A, together with T188I (not present in A/Alberta/01/2014), increased binding to α2,6 SA by pseudotyping

Figure 2. Neighbor-joining phylogenetic tree of the hemagglutinin (HA) genes of clade 2.3.2.1 highly pathogenic avian influenza A(H5N1) viruses with A/Alberta/01/2014 (GISAID accession no. EPI500771). The avian influenza A(H5N1) virus detected in Canada is underlined. The nearest reassortant World Health Organization candidate vaccine viruses (CVV) for each group of clade 2.3.2.1 are denoted by CVV. Asterisks indicated viruses collected in 2012–2014. Amino acid differences at branch nodes indicate HA1 substitutions relative to the nearest CVV for clade 2.3.2.1 viruses (group 2.3.2.1c, A/duck/Vietnam/NCVD-1584/2012; group 2.3.2.1b, A/barn-swallow/HK/D10–1161/2010). Mutations to the right of each strain name indicate amino acid changes found only in that virus relative to the nearest CVV. Bootstraps generated from 1,000 replicates are shown at branch nodes. Scale bar represents nucleotide substitutions per site. Black arrowhead indicates position of clade 2.3.2.1a. GSAID, Global Initiative on Sharing Avian Influenza Data.

and glycan array assays (*8*). When together, S155N and T156A also increased binding to α2,6 SA (assayed with resialated erythrocytes). T156A abrogates a *N*-glycosylation site and, when together with S223N (not found in A/Alberta/01/2014), may improve virus replication in the upper respiratory tract of ferrets (*9*); T156A is consistently found in ferret-adapted mutants capable of airborne transmission (*5*). The collective effects of all these mutations and their phenotypic manifestations are unclear.

Comparison of the HA amino acid sequence of A/Alberta/01/2014 with that of the nearest H5N1 clade 2.3.2.1c World Health Organization candidate vaccine virus (A/duck/Vietnam/NCVD-1584/2012) identified only 2 aa substitutions in the HA1 region, R189K and G221R. Although position 189 was within the putative antigenic site B, the overall conservation of sequence suggests that A/Alberta/01/2014 is a close antigenic match to the candidate vaccine virus.

In agreement with Xu et al. (*4*), no mutations conferring reduced susceptibility to neuraminidase inhibitors were identified for clade 2.3.2.1. The predicted amino acid sequence of the M2 protein did not reveal any changes associated with reduced susceptibility to adamantanes (*10*). Mutation V27I was found, but its significance is uncertain. Mutations N30D and T215A found in the M1 gene of A/Alberta/01/2014 were associated with increased virulence in mice. The cumulative effect of these changes may result in increased lethality (*11*).

The PB2 sequence showed the presence of E627 in both the primary specimen and isolate, establishing the lack of a well-known mammalian adaptation motif (*5*,*12*). Amino acid changes L89V, G309D, T339K, R477G, I495V, and K627E and a change to Met at the predicted position A676T (*13*) were noted in the A/Alberta/01/2014 isolate. These PB2 substitutions in conjunction with changes in the M1 and HA proteins (only some of which were identified) have been described to enhance polymerase activity and virulence in mice. Experiments in mice also demonstrated that compensatory amino acid substitutions in PB2 can rescue polymerase activity in K627E mutants (*13*). Lethal HPAI A(H5N1) isolates, such as A/quail/Vietnam/36/04, show the presence of E627, suggesting that compensatory mutations are possible in PB2 and other genes (*14*). The PB1 protein showed the P598L mutation reported to enhance polymerase activity in mammalian cells and mice (*3*). This change has been reported to enhance the polymerase activity of an attenuated human virus carrying the PB2 K627E mutation (*15*). Of the polymerase mutations hypothesized to increase the RNA polymerase activity of HPAI A(H5N1) viruses, namely P149S, R226H, K357I, and T515S, only two, 149S and 357T, were present in the A/Alberta/01/2014 isolate (*3*).

Mutations in the nucleoprotein gene reported to enhance replication efficiency, virulence, and transmission (*3*) were absent in the isolate. Several NS1 mutations reported to increase virulence in mice were present in A/Alberta/01/2014: P42S, D87E, L98F, and I101M; a 4-bp deletion from nt 80–84, along with the D92E shift; and the PDZ ligand domain (ESEV) at the C terminus (*3*). The multifunctional NS1 protein is a recognized virulence determinant that counters the cellular innate immune response, and the P42S change has been shown to antagonize interferon induction and prevent activation of the nuclear factor–κB and interferon regulatory factor–3 pathways (*16*).

Conclusion

Analysis of the whole genome of HPAI A(H5N1) virus provides valuable insight into the presence of mutations that may reflect adaptive changes, altered virulence, and/or transmission phenotype. Because of the unique manifestation of neurologic symptoms and encephalitis reported in this patient, additional studies are needed to understand the broader aspects of virus heterogeneity and its role in this fatal case.

Acknowledgments

We gratefully acknowledge the tremendous work of the clinical and public health teams in Alberta involved in the management and follow-up of this case and deeply appreciate the cooperation of the family during the investigation of this tragic event. We thank the technical laboratory staff for their work and contributions to the confirmation and analysis of this influenza strain. We greatly appreciate and acknowledge the generous discussions and expert input of Nancy Cox and her team at the US Centers for Disease Control and Prevention.

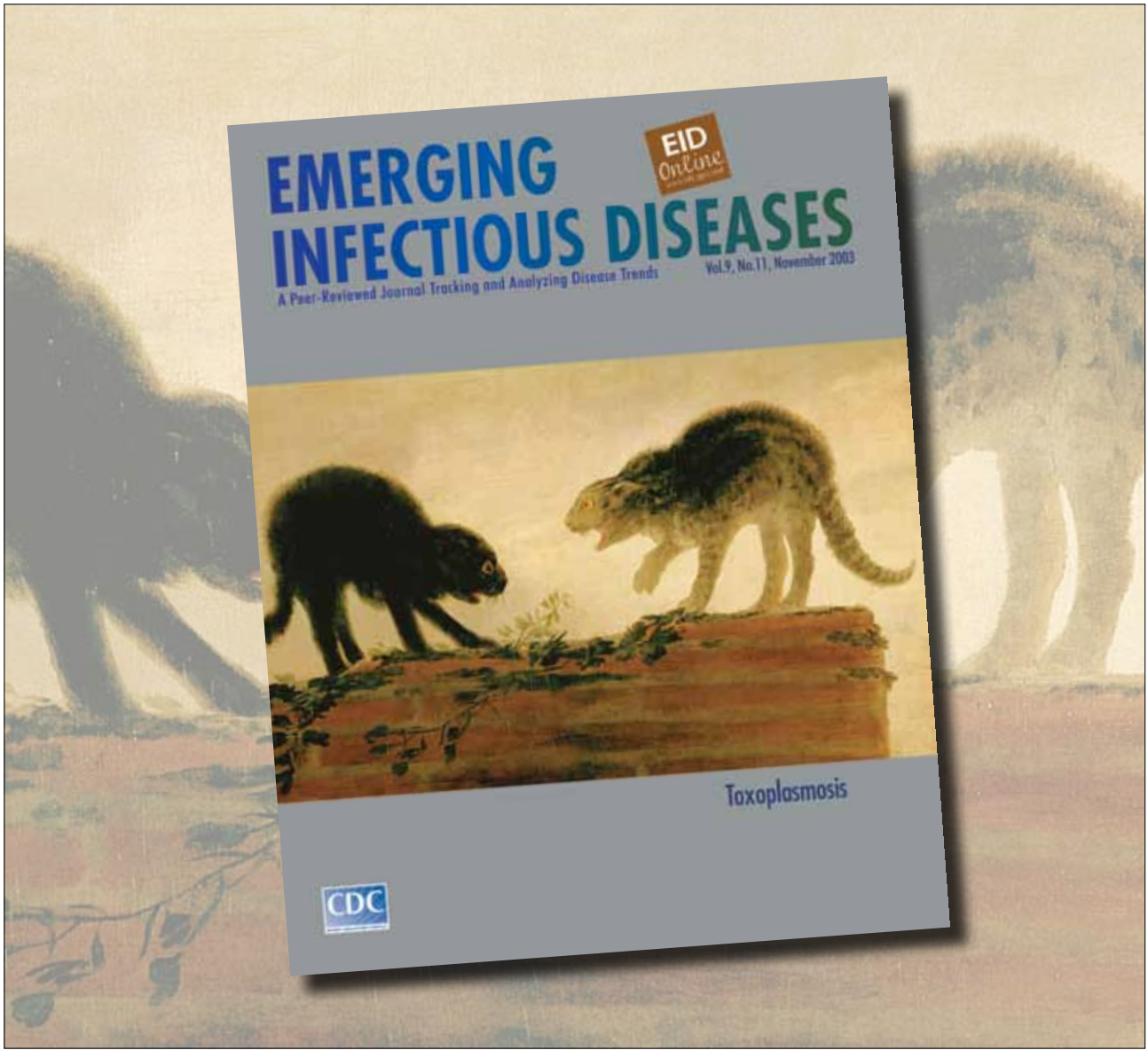
Ms Pabbaraju is a senior laboratory scientist at the Provincial Laboratory for Public Health. Her research focuses on the development of diagnostic tests for viral and bacterial pathogens as well as studies on the epidemiology of viruses.

References

1. ProMED-mail. Fatal avian influenza A(H5N1) infection in a Canadian traveler. 2014 Jan 12 [cited 2014 Jan 24]. http://www.promedmail.org, archive no. 20140112.2167282
2. World Health Organization/World Organization for Animal Health/Food and Agriculture Organization (WHO/OIE/FAO) H5N1 Evolution Working Group, 2013. Revised and updated nomenclature for highly pathogenic avian influenza A (H5N1) viruses. Influenza Other Respir Viruses. 2014. Epub 2014 Jan 31.
3. Centers for Disease Control and Prevention. H5N1 Genetic Changes Inventory: a tool for influenza surveillance and preparedness [cited 2014 Jan 24]. http://www.cdc.gov/flu/avianflu/h5n1-genetic-changes.htm
4. Xu L, Bao L, Yuan J, Li F, Lv Q, Deng W, et al. Antigenicity and transmissibility of a novel clade 2.3.2.1 avian influenza H5N1 virus. J Gen Virol. 2013;94:2616–26. http://dx.doi.org/10.1099/vir.0.057778-0
5. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, et al. Airborne transmission of influenza A/H5N1 virus between ferrets. Science. 2012;336:1534–41. http://dx.doi.org/10.1126/science.1213362
6. Martín J, Wharton SA, Lin YP, Takemoto DK, Skehel JJ, Wiley DC, et al. Studies of the binding properties of influenza hemagglutinin receptor-site mutants. Virology. 1998;241:101–11. http://dx.doi.org/10.1006/viro.1997.8958
7. Su Y, Yang HY, Zhang BJ, Jia HL, Tien P. Analysis of a point mutation in H5N1 avian influenza virus hemagglutinin in relation to virus entry into live mammalian cells. Arch Virol. 2008;153:2253–61. http://dx.doi.org/10.1007/s00705-008-0255-y
8. Yang ZY, Wei CJ, Kong WP, Wu L, Xu L, Smith DF, et al. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. Science. 2007;317:825–8. http://dx.doi.org/10.1126/science.1135165
9. Wang W, Lu B, Zhou H, Suguitan AL Jr, Cheng X, Subbarao K, et al. Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets. J Virol. 2010;84:6570–7. http://dx.doi.org/10.1128/JVI.00221-10

10. Govorkova EA, Baranovich T, Seiler P, Armstrong J, Burnham A, Guan Y, et al. Antiviral resistance among highly pathogenic influenza A (H5N1) viruses isolated worldwide in 2002–2012 shows need for continued monitoring. Antiviral Res. 2013;98:297–304. http://dx.doi.org/10.1016/j.antiviral.2013.02.013
11. Fan S, Deng G, Song J, Tian G, Suo Y, Jiang Y, et al. Two amino acid residues in the matrix protein M1 contribute to the virulence difference of H5N1 avian influenza viruses in mice. Virology. 2009;384:28–32. http://dx.doi.org/10.1016/j.virol.2008.11.044
12. Long JS, Howard WA, Núñez A, Moncorgé O, Lycett S, Banks J, et al. The effect of the PB2 mutation 627K on highly pathogenic H5N1 avian influenza virus is dependent on the virus lineage. J Virol. 2013;87:9983–96. http://dx.doi.org/10.1128/JVI.01399-13
13. Li J, Ishaq M, Prudence M, Xi X, Hu T, Liu Q, et al. Single mutation at the amino acid position 627 of PB2 that leads to increased virulence of an H5N1 avian influenza virus during adaptation in mice can be compensated by multiple mutations at other sites of PB2. Virus Res. 2009;144:123–9. http://dx.doi.org/10.1016/j.virusres.2009.04.008
14. Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, Hulse-Post DJ, et al. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med. 2006;203:689–97. http://dx.doi.org/10.1084/jem.20051938
15. Xu C, Hu WB, Xu K, He YX, Wang TY, Chen Z, et al. Amino acids 473V and 598P of PB1 from an avian-origin influenza A virus contribute to polymerase activity, especially in mammalian cells. J Gen Virol. 2012;93:531–40. http://dx.doi.org/10.1099/vir.0.036434-0
16. Jiao P, Tian G, Li Y, Deng G, Jiang Y, Liu C, et al. A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J Virol. 2008;82:1146–54. http://dx.doi.org/10.1128/JVI.01698-07

Address for correspondence: Kevin Fonseca, Provincial Laboratory for Public Health, 3030 Hospital Dr NW, Calgary, AB T2N 4W4, Canada; email: kevin.fonseca@albertahealthservices.ca



Who Is This Person?

She studied the viral etiology of cancer and identified the murine polyomavirus.

Is she:

A) Sarah Stewart

B) Janet Rowley

C) Charlotte Friend

D) Bernice Eddy

Decide first. Then turn the page.

Sarah Elizabeth Stewart

Carl Fulghieri and Sharon Bloom

This is a photograph of Sarah Elizabeth Stewart, PhD, MD (1905–1976), whose discoveries involving the murine polyomavirus with Bernice Eddy, PhD, propelled the then-reluctant field of oncology to pursue viral etiologies of cancer. Sarah Stewart was born on August 16, 1905, in Jalisco, Mexico, to an American engineer and Mexican mother. After moving with her family back to the United States at age 5, Stewart remained fluent in Spanish. In 1927, she graduated from New Mexico State University and in 1930, she earned an MS in Microbiology at the University of Massachusetts, Amherst. She worked at the National Institutes of Health (NIH) during 1935–1944, publishing 7 papers on anaerobic bacteria and completing a PhD from the University of Chicago in 1939.

In 1944, when Stewart requested support to study the link between animal tumors and viruses, the directors of the NIH Laboratory of Microbiology and the National Cancer Institute (NCI) refused on the grounds that the proposal seemed dubious and that she lacked appropriate qualifications. Rather than give up her passion to study viruses and cancer, Stewart resigned and became a bacteriology instructor at Georgetown University School of Medicine; there she audited classes, and after women were permitted to enroll, Stewart became their first woman graduate at age 39. After completing an internship in 1951, Stewart returned to NCI to launch her viral oncology research career.

To understand Stewart's role in catalyzing viral oncology research, it is necessary to recognize that until the 1950s, scientists dismissed the idea that viruses could cause cancer. It took many decades before the seminal contributions of several virologists studying cancers were appreciated, such as Peyton Rous' 1911 discovery of the Rous sarcoma virus (which caused tumors in chickens), and discoveries of Richard Shope (rabbit fibroma) and John Bittner (mouse mammary carcinoma) in the 1930s. In 1951, Ludwig Gross described the transmission of leukemia in newborn mice by using a cell-free extract; in 1953, he reported parotid tumors in these mice. Even after Stewart confirmed Gross's findings in 1953, the scientific community still did not acknowledge viral causes of mammalian cancer. Only after Stewart fulfilled Koch's postulates in 1957, with the assistance of Dr. Bernice Eddy, did oncologists pay heed to viruses.

Dr. Eddy had also trained as a PhD bacteriologist at the University of Chicago and since 1937, worked at the NIH Biologics Control Laboratories, in Bethesda, Maryland. In 1954, Eddy had been sidelined for whistleblowing about the presence of live virus in Jonas Salk's inactivated polio vaccine (the infamous Cutter incident). So in 1956, when

Figure. Sarah Elizabeth Stewart (1905–1976). Source: National Library of Medicine, Hobson-Huntsinger University Archives at New Mexico State University.

Author affiliations: Carrboro High School, Carrboro, North Carolina, USA (C. Fulghieri); and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (S. Bloom)

DOI: http://dx.doi.org/10.3201/eid2005.131876

Stewart approached Eddy for assistance growing the agent causing parotid tumors in mice, Eddy readily agreed and the 2 women rapidly worked out the characteristics of the agent that was not referred to as a virus in their publications until 1959. Together they showed that the virus produced 20 types of mouse tumors and could cause tumors in other small mammals. At Eddy's suggestion, the virus was dubbed polyoma, which means many tumors, and they named it the SE (Stewart–Eddy) polyomavirus. They also demonstrated that the virus causes cell necrosis and proliferation in cell culture, that it is highly antigenic, and that it leads to formation of specific antibodies in infected animals whether or not tumors develop.

The results of their collaboration were picked up by a 1959 Time Magazine cover story, citing John Heller, then the NCI director, "the hottest thing in cancer research is research on viruses as possible causes of cancer." Alan Rabson, a prominent member of the NCI Laboratory of Pathology, stated, "The whole place just exploded after Sarah found polyoma."

Stewart became medical director of the NCI Laboratory of Oncology and spent the remainder of her life researching several oncogenic viruses (e.g., Epstein-Barr virus). As a US Public Health Service Commissioned Officer, her scientific contributions to the study of viral etiologies of cancer earned her the Federal Women's Award, presented by President Lyndon Johnson in 1965. In 1960, Eddy again found herself in hot water, this time for reporting her discovery of an oncogenic simian virus (SV40) in polio vaccine prepared from monkey kidneys. Stewart retired from the Public Health Service in 1970 to become a full professor of pathology at Georgetown University. She died of stomach cancer in 1976. Bernice Eddy described her as "a forceful individual who did not let anything stand in [her] way if she could help it." Despite sex discrimination and a period in which several laboratory directors disparaged her wish to study oncogenic viruses, Stewart persisted with such enthusiasm that she managed to break through as one of the most influential scientists and cancer researchers of her time.

Mr Fulghieri is a junior attending Carrboro High School, Carrboro, North Carolina. He got the idea to submit a Photo Quiz after attending the Centers for Disease Control and Prevention Disease Detective Camp for high school students.

Dr Bloom is an associate editor for Emerging Infectious Diseases.

Suggested Reading

1. O'Hern EM. Sarah Elizabeth Stewart. In: Profiles of pioneer women scientists. Washington (DC): Acropolis; 1985. p. 161–9.
2. Stewart SE. Leukemia in mice produced by a filterable agent present in AKR leukemic tissues with notes on a sarcoma produced by the same agent [abstract]. Anat Rec. 1953;117:532.
3. Stewart SE. Neoplasms in mice inoculated with cell-free extracts or filtrates of leukemic mouse tissue. I. Neoplasms of the parotid and adrenal glands. J Natl Cancer Inst. 1955;15:1391–415 .
4. Eddy BE, Stewart SE, Stanton MF, Marcotte JM. Induction of tumors in rats by tissue-culture preparations of SE polyoma virus. J Natl Cancer Inst. 1959;22:161–71 .
5. Gross L. Oncogenic viruses. 2nd ed. New York: Pergamon; 1970.
6. Shimkin MB. As memory serves: an informal history of the National Cancer Institute, 1937–57. J Natl Cancer Inst. 1977;59 (suppl):559–600.
7. Howley PM, Livingston DM. Small DNA tumor viruses: large contributors to biomedical sciences. Virology. 2009;384:256–9. http://dx.doi.org/10.1016/j.virol.2008.12.006
8. Murphy FA. The foundations of virology. West Conshohocken (PA): Infinity; 2013. p. 274.
9. Shorter E. Cancer. In: The health century. New York: Doubleday; 1987. p. 179–211.

Address for correspondence: Sharon Bloom, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E41, Atlanta, GA 30333, USA; email: sbloom@cdc.gov

Babesia venatorum Infection in Child, China

To the Editor: Babesiosis, which is caused by intraerythrocytic sporozoites of the genus *Babesia*, is a tick-borne emerging zoonosis in humans. Although >100 *Babesia* species infect animals, only a few species, mainly *B. microti* and *B. divergens*, infect humans. Human infections with *B. microti* have been reported from the United States and other countries, and most human infections with *B. divergens* have been reported from Europe (*1*). Another species, *B. venatorum*, was found to infect humans in some countries in Europe (*2*,*3*).

Only 12 babesiosis case-patients have been reported in China, 10 of whom were infected with *B. microti* (*4*–*6*) and 2 with *B. divergens* (*7*). We report a case of babesiosis caused by *B. venatorum* in a child and characterize the isolated pathogen.

On April 16, 2012, an 8-year-old boy who lived in Pishan County, Xinjiang Autonomous Region, China, was admitted to Friendship Hospital in Beijing because of an irregular fever (38.6°C–41.0°C) for 12 days, anemia, malaise, myalgia, fatigue, progressive weakness, and shortness of breath. Before admission, he was given oral cefixime (80 mg/day for 5 days) at a local clinic, but no clinical improvement was observed.

At admission, the patient had a body temperature of 38.7°C, a pulse rate of 76 beats/min, a blood pressure of 110/70 mm Hg, and a respiration rate of 18 breaths/min. Laboratory tests identified hemoytic anemia (erythrocyte count 2.7×10^9 cells/L, hemoglobin level 8.6 g/dL), thrombocytopenia (147×10^9 platelets/L), increased levels of serum lactate dehydrogenase (1,462 U/L) and bilirubin (2.6 mg/dL), and an increased leukocyte count (17×10^9 cells/L with 72% neutrophils, 24% lymphocytes, 1% monocytes, 2% eosinophils, and 1% basophils). Levels of C-reactive protein (14.2 mg/dL) and procalcitonin (3.1 mg/dL) were increased, which suggested an inflammatory process.

Forty-eight hours after the patient's admission, a thin peripheral blood smear stained with Giemsa was prepared. A presumptive diagnosis of babesiosis was made on the basis of microscopic observation of intraerythrocytic parasites (parasitemia level ≈5%) with typical ring-like trophozoites, paired pyriforms, and tetrads (Figure, panel A).

DNA was extracted from a patient blood sample. PCR specific for a partial 18S rRNA gene sequence was performed with primers PIRO-A and PIRO-B (*8*) and showed a positive result for a *Babesia* sp. The patient was then treated with azithromycin (12 mg/kg once a day for 7 days) and atovaquone (20 mg/kg twice a day for 7 days). His clinical manifestations improved 3 days after treatment, although parasites were still detectable in blood smears. On May 17, negative results for blood smears and PCR indicated that the parasite had been cleared. The boy was discharged on May 20, 2012, and has remained healthy.

A 0.5-mL blood sample obtained from the patient before treatment was injected intraperitoneally into 3 severely combined immunodeficient mice. Mice were monitored for parasitemia every 3 days. When tested 6–9 days postinjection, all 3 mice were positive for a *Babesia* sp. (Figure, panel B). We tested for IgG against *B. venatorum* and *B. microti* by using an indirect immunofluorescence assay (*9*). Seroconversion against *B. venatorum* was evident; reciprocal antibody titers of 16 in an acute-phase sample (admission) and 128 in a convalescent-phase (discharge) sample. Results for *B. microti* were negative.

Nucleotide sequences of PCR products from patient and mice blood samples were identical to the corresponding sequence of *B. venatorum* 18S rRNA. The complete 18S rRNA gene of the *Babesia* parasite isolated from the patient was amplified with primers CRYPTOF and CRYPTOR (*2*). This PCR product was sequenced, and the sequence was submitted to GenBank under accession no. KF724377.

B. venatorum was first known as *Babesia* sp. EU1 and was named after the Latin word for hunter because the first reported infected patients were 2 occupational hunters from Austria and Italy (*2*). One human infection with *B. venatorum* was also reported from Germany (*3*). All 3 case-patients were men >50 years of age who had undergone splenectomies for severe Hodgkin disease before *Babesia* sp. infection.

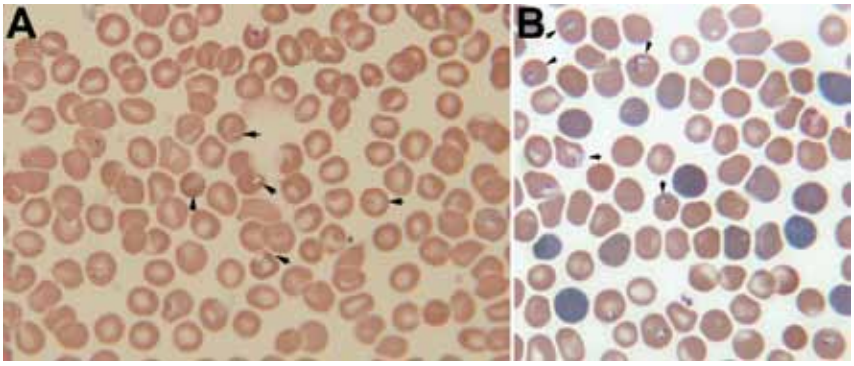


Figure. A) Giemsa-stained thin blood smear for an 8-year-old boy from China showing erythrocytes with typical ring forms, paired pyriforms, and tetrads of a *Babesia* sp. (arrows). B) Giemsa-stained thin blood smear for a mouse with severely combined immunodeficiency, which had been injected with blood from the patient, showing *Babesia* sp.–infected erythrocytes (arrows). Original magnifications × 1,000.

Previously reported babesiosis cases in children have been mostly acquired by blood transfusion (*10*). The patient had no history of transfusions with blood products and had never traveled outside his home town before disease onset. Although he and his parents did not recall any tick bites, he was at high risk for exposure to ticks because he often played with his dog, which frequently went outdoors in a tick-infested forested area. The dog may have transmitted a *Babesia* sp.–infected tick to the patient. However, ticks from the dog were not available for identification and testing.

The patient in our study was presumed to be healthy and immunocompetent, which indicates that *Babesia* species can cause infections even in healthy persons. Babesiosis should be considered in the differential diagnosis of patients with a history of tick exposure and prolonged and irregular fever. Blood smear evaluation for intraerythrocytic parasites should be considered.

The patient was treated with azithromycin and atovaquone and the parasites were cleared within 1 month. This combined treatment was well tolerated and effective, and it can be recommended as an alternative treatment to the commonly used therapy of quinine and clindamycin (*1*).

This study was supported by the Natural Science Foundation of China (grants 81130086, 30400364, and 30872196) and the Special Fund for Health Research in the Public Interest (grant 201202019).

Yi Sun, Shao-Gang Li, Jia-Fu Jiang, Xin Wang, Yuan Zhang, Hong Wang, and Wu-Chun Cao

Author affiliations: Beijing Institute of Microbiology and Epidemiology, Beijing, China (Y. Sun, J.-F. Jiang, X. Wang, Y. Zhang, H. Wang, W.-C. Cao); and Capital Medical University, Beijing (S.-G. Li)

DOI: http://dx.doi.org/10.3201/eid2005/121034

References

1. Vannier E, Krause PJ. Human babesiosis. N Engl J Med. 2012;366:2397–407. http://dx.doi.org/10.1056/NEJMra1202018
2. Herwaldt BL, Cacciò S, Gherlinzoni F, Aspöck H, Slemenda SB, Piccaluga P, et al. Molecular characterization of a non–*Babesia divergens* organism causing zoonotic babesiosis in Europe. Emerg Infect Dis. 2003;9:942–8. http://dx.doi.org/10.3201/eid0908.020748
3. Häselbarth K, Tenter AM, Brade V, Krieger G, Hunfeld KP. First case of human babesiosis in Germany: clinical presentation and molecular characterisation of the pathogen. Int J Med Microbiol. 2007;297:197–204. http://dx.doi.org/10.1016/j.ijmm.2007.01.002
4. Su GG, Zhao NF, Ye XX. A case report of babesiosis [in Chinese]. Chinese Journal of Zoonoses. 2002;18:112.
5. Yao LN, Wei R, Zeng CY, Li ZH, Zhang X, Lei Y, et al. Pathogen identification and clinical diagnosis for one case infected with *Babesia* [in Chinese]. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 2012;30:118–21.
6. Zhou X, Li SG, Chen SB, Wang JZ, Xu B, Zhou HJ, et al. Co-infections with *Babesia microti* and *Plasmodium* parasites along the China–Myanmar border. Infect Dis Poverty. 2013;2:24. http://dx.doi.org/10.1186/2049-9957-2-24
7. Qi C, Zhou D, Liu JZ, Cheng ZQ, Zhang L, Wang L, et al. Detection of *Babesia divergens* using molecular methods in anemic patients in Shandong Province, China. Parasitol Res. 2011;109:241–5. http://dx.doi.org/10.1007/s00436-011-2382-8
8. Armstrong PM, Katavolos P, Caporale DA, Smith RP, Spielman A, Telford SR III. Diversity of *Babesia* infecting deer ticks (*Ixodes dammini*). Am J Trop Med Hyg. 1998;58:739–42.
9. Hunfeld KP, Lambert A, Kampen H, Albert S, Epe C, Brade V, et al. Seroprevalence of *Babesia* infections in humans exposed to ticks in midwestern Germany. J Clin Microbiol. 2002;40:2431–6. http://dx.doi.org/10.1128/JCM.40.7.2431-2436.2002
10. Fox LM, Wingerter S, Ahmed A, Arnold AP, Chou J, Rhein L, et al. Neonatal babesiosis: case report and review of the literature. Pediatr Infect Dis J. 2006;25:169–73. http://dx.doi.org/10.1097/01.inf.00001 95438.09628.b0

Address for correspondence: Wu-Chun Cao, State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology. 20 Dongda St, Fengtai District, Beijing 100071, China; email: caowc@bmi.ac.cn

Extended-Spectrum β-Lactamases in *Escherichia coli* and *Klebsiella pneumoniae* in Gulls, Alaska, USA

To the Editor: Resistance to β-lactam antibacterial drugs has spread rapidly, particularly through the CTX-M β-lactamase enzymes (CTX-M) (*1*). Although CTX-Ms are geographically widely distributed, reports of extended-spectrum β-lactamase (ESBL) dissemination are few from remote regions. In 2008, we reported phenotypic resistance traits in *Escherichia coli* isolates in 8.2% of wild birds sampled in the Arctic (*2*). We sampled approximately 260 wild birds, mainly gulls and geese, but found no ESBL-harboring isolates (J. Bonnedahl et al., unpub. data). Here we report results of our 2010 study at Barrow, Alaska, USA, a follow up to our 2005 study in which we found vancomycin-resistant enterococci (VRE) with clear traits of human origin in glaucous gulls (*3*). Our findings show a remarkable change, not in VRE dissemination, which is fairly unchanged, but in the emergence of ESBLs and general resistance of *E. coli* isolates.

We collected 150 fecal samples from a population of adult gulls residing close to a landfill site. For a description of general resistance levels (*4*,5), susceptibility of 1 randomly selected *E. coli* isolate per sample (137 isolated from 150 samples) was tested to a set of 10 antibacterial agents. Nearly half (48%) of the 137 *E. coli* isolates were resistant to at least 1 of the drugs tested. Resistance to 1 or 2 antimicrobial agents was found in 32% and 13% of the tested isolates, respectively, and resistance to ≥3 was found in 3% of isolates (online Technical Appendix Table, wwwnc.cdc.gov/EID/article/20/5/13-0325-Techapp1.pdf).

We analyzed samples for presence of VRE (*3*). Seven (4.7%) *E. faecium* isolates were found, all of which harbored both the *vanA* and the *esp* genes (found in isolates of the CC17 lineage) (*3*). No other VRE were found.

To investigate the presence of ESBL-producing bacteria, we conducted a selective screen as described (*6*). ESBL-producing bacteria were found (*E. coli* and *K. pneumoniae*), and ESBL genes ($bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM}) in ESBL-positive isolates were analyzed (*6*). We found 33 *E. coli* and 35 *K. pneumoniae* ESBL-producing isolates in 55 samples (12 samples had >1 unique isolate), a total of 37% of ESBL-harboring samples (Table).

We performed multi-locus sequence typing (MLST) on ESBL-producing *E. coli* isolates (*4*). Isolates were of described sequence types (STs) (ST131 [12 isolates], ST38 [10], ST405 [3], and ST10 [1]), and of previously undescribed STs (designated ST2253 [1 isolate] and ST2967 [6 isolates]) (Table).

In our 2005 study in Barrow, general resistance was relatively low, and no ESBL was found; surprisingly, however, 2 VRE isolates of a human clonal lineage were found (*3;* M. Drobni et al., unpub. data). Since then, resistance dissemination, particularly that of ESBLs, has exploded globally (*1*). In 2010, we found a high level of general resistance; 48% of randomly selected *E. coli* isolates displayed resistance toward ≥1 antibacterial drugs. This level is similar to the level we found in gulls in France in 2008, an area with high current and historical clinical antibacterial drug use and where birds have close contact with human activities (*4*).

We screened samples for VRE and ESBL-producing bacteria. The prevalence of VRE decreased from 6% in 2005 to 4.7% in the current study (*3*), indicating a slow decline or stability in VRE. ESBL, on the other hand, was not found in the 2005 study (M. Drobni et al., unpub. data) but emerged in 37% of samples carrying *E. coli* and/or *K. pneumoniae* harboring ESBLs. In the study from France, only 9.4% of birds carried ESBLs (*4*), although a study of gulls in Portugal during 2007–2008 reported an ESBL carriage of 32% (*7*), more similar to results of our current study but in contrast also because they investigated gulls from a highly populated area.

E. coli isolates mainly carried $bla_{\text{CTX-M-14}}$ or $bla_{\text{TEM-19}}$, whereas *K. pneumoniae* isolates mainly carried $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$, or $bla_{\text{SHV-102}}$. To our knowledge, ESBLs in *E. coli* and *K. pneumoniae* have not been reported from Alaska, but in two 10-year perspective reports from Canada (*8*,*9*), similar patterns and genotypes are reported in *E. coli* and *K. pneumoniae* in clinical isolates (mainly from samples of persons with urinary tract infections and urosepsis). Our MLST of *E. coli* indicated 4 known STs; ST10, ST38, ST131, and ST405, all very common in the material from Canada (*8*), and major STs responsible for CTX-M dissemination worldwide (*1*). Two novel STs were found; several isolates were designated to 1 of them. We conclude that the relatively limited variation in clonal variants (STs) and ESBL genotypes is a consequence of recent introduction from connecting areas, such as Canada, possibly directly by bird migration or human activities, of a few resistant clones, followed by a local clonal expansion. This conclusion is supported by our 2005 study showing no ESBLs and by studies showing where different clones might have been introduced continuously for long periods, such as our study in France (*4*), which display a much larger diversity.

The dissemination of ESBLs to Barrow is part of this global pattern, and it is safe to say that humans and wildlife share resistant *E. coli* flora. When areas such as remote parts of Alaska are affected, global coverage is imminent.

This work was supported financially by the Swedish Research Council (2008-6892); the Health Research Council of Southeast Sweden; and the Department of Medical Sciences, Uppsala University.

Table. Characterization of *Escherichia coli* and *Klebsiella pneumoniae* ESBL-producing isolates, Barrow, Alaska, USA*

Isolates, no.†	*bla* genotype			MLST profile
	CTX-M	SHV	TEM	
E. coli				
12	14	–	1	ST38 (ST2253)‡
11	14	–	–	ST131 (ST10)§
5	–	–	19	ST2967¶
3	27	–	–	ST405
1	15	–	–	ST131
1	–	–	1	ST2967¶
K. pneumoniae				
4	15	12	1	ND
5	–	12	1	ND
2	–	12	–	ND
2	–	102	19	ND
8	–	102	–	ND
1	–	–	19	ND
4	15	1	1	ND
1	–	2	–	ND

*ESBL, extended-spectrum β-lactamase; MLST, multilocus sequence type; ST, sequence type; ND, no data.
†*E. coli* comprised 33 isolates from 32 samples. *K. pneumoniae* comprised 35 isolates from 35 samples. and 12 samples were both *E. coli* and *K. pneumoniae* ESBL-harboring isolates but did not display horizontal transfer resulting from deviating resistance genotypes.
‡One of the isolates harbored a novel MLST allele, giving the novel ST2253 (deposited in the *E. coli* MLST database at the ERI, University College, Cork, Ireland. (http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli/).
§One of the isolates had ST10; the remaining 10 had ST131.
¶Isolates harbored a novel MLST allele, rendering the novel ST2967 (deposited in the MLST database). The single isolate with only $bla_{\text{TEM-1}}$ may contain undetected ESBL genes because of the non-ESBL phenotype of TEM-1.

Jonas Bonnedahl, Jorge Hernandez, Johan Stedt, Jonas Waldenström, Björn Olsen, and Mirva Drobni

Author affiliations: Linnaeus University, Kalmar, Sweden (J. Bonnedahl, J. Hernandez, J. Stedt, J. Waldenström); Kalmar County Hospital, Kalmar (J Bonnedahl, J. Hernandez); and Uppsala University, Uppsala, Sweden (J. Hernandez, B. Olsen, M. Drobni)

DOI: http://dx.doi.org/10.3201/eid2005.130325

References

1. Naseer U, Sundsfjord A. The CTX-M conundrum: dissemination of plasmids and *Escherichia coli* clones. Microb Drug Resist. 2011;17:83–97. http://dx.doi.org/10.1089/mdr.2010.0132
2. Sjölund M, Bonnedahl J, Hernandez J, Bengtsson S, Cederbrant G, Pinhassi J, et al. Dissemination of multidrug-resistant bacteria into the Arctic. Emerg Infect Dis. 2008;14:70–2. http://dx.doi.org/10.3201/eid1401.070704
3. Drobni M, Bonnedahl J, Hernandez J, Haemig P, Olsen B. Vancomycin-resistant enterococci, Point Barrow, Alaska, USA. Emerg Infect Dis. 2009;15:838–9. http://dx.doi.org/10.3201/eid1505.081219
4. Bonnedahl J, Drobni M, Gauthier-Clerc M, Hernandez J, Granholm S, Kayser Y, et al. Dissemination of *Escherichia coli* with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. PLoS ONE. 2009;4:e5958. http://dx.doi.org/10.1371/journal.pone.0005958
5. Gordon DM. Geographical structure and host specificity in bacteria and the implications for tracing the source of coliform contamination. Microbiology. 2001;147:1079–85.
6. Bonnedahl J, Drobni P, Johansson A, Hernandez J, Melhus Å, Stedt J, et al. Characterization, and comparison, of human clinical and black-headed gull (*Larus ridibundus*) extended-spectrum beta-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden. J Antimicrob Chemother. 2010;65:1939–44. http://dx.doi.org/10.1093/jac/dkq222
7. Simões RR1, Poirel L, Da Costa PM, Nordmann P. Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. Emerg Infect Dis. 2010;16:110–2. http://dx.doi.org/10.3201/eid1601.090896
8. Peirano G, van der Bij AK, Gregson DB, Pitout JD. Molecular epidemiology over an 11-year period (2000 to 2010) of extended-spectrum β-lactamase–producing *Escherichia coli* causing bacteremia in a centralized Canadian region. J Clin Microbiol. 2012;50:294–9. http://dx.doi.org/10.1128/JCM.06025-11
9. Peirano G, Sang JH, Pitondo-Silva A, Laupland KB, Pitout JD. Molecular epidemiology of extended-spectrum-β–lactamase-producing *Klebsiella pneumoniae* over a 10 year period in Calgary, Canada. J Antimicrob Chemother. 2012;67:1114–20. http://dx.doi.org/10.1093/jac/dks026

Address for correspondence: Mirva Drobni, Department of Medical Sciences/Section of Clinical Microbiology and Infectious Diseases, Uppsala University Hospital, SE-751 85 Uppsala, Sweden; email: mirva.drobni@medsci.uu.se

Staphylococcus aureus Carrying *mec*C Gene in Animals and Urban Wastewater, Spain

To the Editor: A new methicillin resistance mechanism gene, a divergent *mec*A homologue named *mec*C (formerly *mec*A_{LGA251}), was recently described in *Staphylococcus aureus* (*1*). Methicillin-resistant *S. aureus* (MRSA) isolates carrying *mec*C have been recovered from humans, ruminants, pets, and other animals such as rats, seals, and guinea pigs (*1–3*). It has been suggested that *mec*C-carrying MRSA isolates might not be detected by using MRSA selective media (*4*). For *mec*C-carrying *S. aureus* isolates, cefoxitin MICs of 4–64 mg/L have been demonstrated (*1–2*,*4*), values that would normally include susceptible isolates, according to the epidemiologic cutoff value established by the European Committee on Antibiotic Susceptibility Testing (EUCAST; www.eucast.org). *mec*C-carrying *S. aureus* isolates have been classified as heteroresistant (*5*), and MICs can be affected by the drug-susceptibility testing method used (*1,5*).

These observations led us to retrospectively investigate the presence of *mec*C gene in a set of 361 *mec*A-negative *S. aureus* isolates collected during 2009–2012 (Table), independently of their susceptibility to cefoxitin. Isolates were recovered from healthy carriers in livestock (n = 39), from wild animals (n = 254), and from wastewater (effluents) from an urban sewage plant (n = 68). Specific amplification of the *mec*C gene was performed as described (*6*). The *mec*C-carrying *S. aureus* isolates were tested by broth microdilution using Microtiter EUST plates (Trek Diagnostic Systems, East Grinstead, UK) for susceptibility to benzylpenicillin, cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, florfenicol, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, rifampin, sulfamethoxazole, streptomycin, quinupristin-dalfopristin, tetracycline, thiamulin, trimethoprim, and vancomycin. Additionally, susceptibility to oxacillin was determined by using microScan Gram Positive Combo panel 37 (Siemens, Erlangen, Germany). MICs were interpreted according to EUCAST epidemiologic cutoff values.

*mec*C was detected in a total of 4 isolates from wild boar (n = 1), fallow deer (n = 2), and urban wastewater (n = 1); these isolates represent 1% of the 361 tested isolates. The 3 isolates recovered from animals were susceptible to all antimicrobial drugs tested other than β-lactams and to oxacillin (MICs 0.5–1 mg/L) but were resistant to penicillin (MICs 0.5–2 mg/L). Two of the isolates were resistant to cefoxitin (MICs 8 and 16 mg/L) and the third was susceptible (MIC 4 mg/L). The wastewater isolate was resistant to penicillin (MIC 2 mg/L) and erythromycin (MIC 16 mg/L) and susceptible to all other antimicrobial drugs tested, including cefoxitin (MIC 4 mg/L) and oxacillin (MIC ≤0.25 mg/L).

Table. Testing of *Staphylococcus aureus* isolates for presence of methicillin resistance mechanism gene *mec*C, Spain*

Isolate source	Year(s) of isolation	No. *mec*C-positive isolates	*spa* type	MLST	CC	Antimicrobial resistance profile
Livestock, n = 39						
Cattle, n = 5	2011	0				
Fattening pigs, n = 34	2009, 2011	0				
Wild animals, n = 254						
Eurasian griffon vulture, n = 2	2011	0				
Fallow deer, n = 2	2012	2	t11212	ST425	CC425	PEN, FOX
			t11212	ST425	CC425	PEN
Iberian ibex, n = 39	2009–2010	0				
Mouflon, n = 2	2009	0				
Red deer, n = 61	2009–2011	0				
Wild boar, n = 148	2009–2011	1	t11212	ST425	CC425	PEN, FOX
Urban wastewater, n = 68	2011	1	t843	ST2676	CC130	PEN, ERY

*MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex; PEN, benzylpenicillin; FOX, cefoxitin; ERY, erythromycin.

Previous studies have described *mec*C-positive isolates as susceptible to all antimicrobial drugs tested except β-lactams (*2,3*), although sporadic resistance to fluoroquinolones has been found (*4,7*). We additionally found erythromycin resistance in 1 *mec*C-carrying *S. aureus* isolate. For the 4 *mec*C-carrying *S. aureus* isolates we detected, MICs of oxacillin were interpreted as susceptible, and 2 isolates were susceptible to cefoxitin according to EUCAST guidelines, findings that agree with previous reports (*1–2,4*). Thus, *mec*C presence is not always linked to resistance phenotypes for cefoxitin or oxacillin; such unclear findings could hinder the detection of *mec*C-carrying isolates.

We further characterized the 4 *mec*C-carrying *S. aureus* isolates by *spa* typing and detection of Panton-Valentin leukocidin (PVL) toxin genes (*6,8*). Multilocus sequence typing (MLST) was performed according to Enright et al. (*9*) by using self-designed primers *arc* (down 5′-CGATTTGTTGTTGATTAGGTTC-3′), *tpi* (up 5′-CATTAGCAGATTTAGGCGTTA-3′), and *yqi*L (down 5′-GATTGGYTCACCTTTRCGTTG-3′). All 4 isolates were PVL negative. The 3 animal isolates were assigned to a new *spa* type (t11212) and to clonal complex (CC) 425 and sequence type (ST) 425 (Table). ST425 has been previously associated with *mec*C-carrying *S. aureus* isolates in cattle and humans (*1–2*); the animals we sampled were from a game estate and may have had contact with cattle and with urban wastewater. The wastewater isolate was assigned to *spa* type t843 and to a new allelic profile, ST2676, in CC130 (Table). ST2676 represents a single-locus variant of ST130 carrying a different allele for the gene *aro*E. MRSA isolates of CC130 have been associated with humans and animals (*1–4,6*). This result indicates that *mec*C-carrying *S. aureus* isolates can be found in urban wastewater, which may act as an environmental reservoir, as has been demonstrated for *mec*A-carrying *S. aureus* (*10*).

In conclusion, we detected the methicillin resistance mechanism gene *mec*C in nonclinical *S. aureus* isolates from animals and urban wastewater in Spain. Although our data indicate that the frequency of this resistance mechanism is low, this gene appears to be expanding to new areas. Prospective studies should be performed to evaluate epidemiologic changes and to analyze the genetic lineages that carry this resistance mechanism.

M. Concepción Porrero, Aránzazu Valverde, Pedro Fernández-Llario,[1] Alberto Díez-Guerrier, Ana Mateos, Santiago Lavín, Rafael Cantón, José-Francisco Fernández-Garayzabal, and Lucas Domínguez

Author affiliations: Universidad Complutense, Madrid, Spain (M.C. Porrero, A. Valverde, A. Díez-Guerrier, A. Mateos, J.-F. Fernández-Garayzabal, L. Domínguez); Universidad de Extremadura, Cáceres, Spain (P. Fernández-Llario); Universitat Autònoma de Barcelona, Bellaterra, Spain (S. Lavín); and Hospital Universitario Ramón y Cajal and Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid (R. Cantón)

DOI: http://dx.doi.org/10.3201/eid2005.130426

References

1. García-Álvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, Curran MD, et al. Meticillin-resistant *Staphylococcus aureus* with a novel *mec*A homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect Dis. 2011;11:595–603. http://dx.doi.org/10.1016/S1473-3099(11)70126-8
2. Paterson GK, Larsen AR, Robb A, Edwards GE, Pennycott TW, Foster G, et al. The newly described *mec*A homologue, mecALGA251, is present in methicillin-resistant *Staphylococcus aureus* isolates from a diverse range of host species. J Antimicrob Chemother. 2012;67:2809–13. http://dx.doi.org/10.1093/jac/dks329
3. Walther B, Wieler LH, Vincze S, Antao EM, Brandenburg A, Stamm I, et al. MRSA variant in companion animals. Emerg Infect Dis. 2012;18:2017–20. http://dx.doi.org/10.3201/eid1812.120238
4. Cuny C, Layer F, Strommenger B, Witte W. Rare occurrence of methicillin-resistant *Staphylococcus aureus* CC130 with a novel *mec*A homologue in humans in Germany. PLoS ONE. 2011;6:e24360

[1]Current affiliation: Innovación en Gestión y Conservación de Ungulados S.L., Cáceres, Spain.

http://dx.doi.org/10.1371/journal.pone.0024360.
5. Kim C, Milheirico C, Gardete S, Holmes MA, Holden MT, de Lencastre H, et al. Properties of a novel PBP2A protein homolog from *Staphylococcus aureus* strain LGA251 and its contribution to the beta-lactam-resistant phenotype. J Biol Chem. 2012;287:36854–63. http://dx.doi.org/10.1074/jbc.M112.395962
6. Stegger M, Andersen PS, Kearns A, Pichon B, Holmes MA, Edwards G, et al. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mec*A or the new *mec*A homologue *mec*A(LGA251). Clin Microbiol Infect. 2012;18:395–400. http://dx.doi.org/10.1111/j.1469-0691.2011.03715.x
7. Petersen A, Stegger M, Heltberg O, Christensen J, Zeuthen A, Knudsen LK, et al. Epidemiology of methicillin-resistant *Staphylococcus aureus* carrying the novel *mec*C gene in Denmark corroborates a zoonotic reservoir with transmission to humans. Clin Microbiol Infect. 2013;19:E16–22 http://dx.doi.org/10.1111/1469-0691.12036.
8. Harmsen D, Claus H, Witte W, Rothganger J, Turnwald D, Vogel U. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. J Clin Microbiol. 2003;41:5442–8. http://dx.doi.org/10.1128/JCM.41.12.5442-5448.2003
9. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol. 2000;38:1008–15.
10. Börjesson S, Matussek A, Melin S, Lofgren S, Lindgren PE. Methicillin-resistant *Staphylococcus aureus* (MRSA) in municipal wastewater: an uncharted threat? J Appl Microbiol. 2010;108:1244–51. http://dx.doi.org/10.1111/j.1365-2672.2009.04515.x

Address for correspondence: Lucas Domínguez, Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense, Avenida Puerta de Hierro s/n 28040, Madrid, Spain; email: lucasdo@visavet.ucm.es

Schmallenberg Virus Antibodies in Adult Cows and Maternal Antibodies in Calves

To the Editor: Schmallenberg virus (SBV), a novel orthobunyavirus that is transmitted by *Culicoides* spp. biting midges, spread through herds of ruminants across Europe during 2011–2013. The virus reached as far as Finland in the north, the Republic of Ireland in the west, Turkey in the east (*1*), and Spain in the south. The clinical effect of SBV infection in ruminant livestock appears to be limited (*2*), and a vaccine to prevent the infection has been developed (*3*). There are no data to refute the assumption that natural SBV infection results in long-term immunity, as was seen earlier with natural infection of cattle with bluetongue virus serotype 8 (*4*). Newborn calves acquire passive immunity by ingestion and absorption of antibodies present in colostrum. Passive immunity can, however, block the production of serum antibodies when vaccine is administered to calves that have maternally derived antibodies (*5*). To determine the titers and persistence of SBV antibodies in adult cows and the decay of maternal antibodies in calves over time, we studied a herd of cattle from a dairy farm in the eastern Netherlands during April 2012–April 2013.

The dairy farm is the only location in the Netherlands where monitoring for biting midges was continuously conducted during the 2011–2013 SBV epidemic and where SBV RNA was detected in biting midges caught during 2011–2012 (*6,7*). The dairy herd comprised 110 animals: 60 milking cows (average age 4.0 years) and 50 heifers (average age 1.5 years) and calves (<1.0 year of age). No clinical signs or symptoms of SBV infection were observed in any of the cattle at the end of 2011 or during 2012. However, during the study period, 3 calves were stillborn, none of which had the characteristic malformations observed after SBV infection. Gross pathology confirmed that the calves did not have SBV infection, and all tissue samples were negative for SBV by reverse transcription PCR.

During the 12-month study, we obtained 4 blood samples from all animals in the herd. A virus neutralization test (VNT) was used to test the samples for antibodies (*8*). For optimal specificity and sensitivity, the VNT cutoff dilution was set at 1:8. Test dilutions ranged from 1:4–1:512. All samples were tested in duplicate; titers were determined using the Reed-Münch method and expressed on a $\log_2$ scale.

Blood samples were first obtained from the herd on April 19, 2012, after retrospective detection of SBV RNA in biting midges that had been collected from the farm on September 14, 2011 (*6*). The remaining 3 blood samples for each animal were collected on September 17, 2012; December 9, 2012; and April 23, 2013 (5, 8, and 12 months, respectively, after the first collection). SBV VNT results for the initial blood samples were positive for all cows ≥1 year of age and for all but four 6-month-old calves. One year later, blood samples for 98% of the cows ≥1 year of age and 50% of the cows <1 year of age were SBV seropositive. During the year, the mean $\log_2$ VNT titer of the adult cows dropped from 8.3 to 6.7.

It can be assumed that cows ≥1 year of age became infected with SBV around the time SBV-infected *Culicoides* biting midges were detected on the farm in September 2011 (*6*). Thus, at least 19 months after natural infection, these cows were probably protected against SBV when re-exposed to the virus. Of all cattle tested, 11 heifers seroconverted between April 2012 and September 2012, and 1 cow seroconverted between the September and December 2012 samplings. The low rate of seroconversion was matched by a 6× lower

proportion of SBV-infected *Culicoides* midges found in 2012 than in 2011 (*7*). We also assume that the level of SBV circulating in the area during 2012 was lower than that in 2011.

Blood samples obtained from 13 calves ≤30 days after birth had a mean $\log_2$ SBV VNT titer of 8.0 (range 6.5–9.5) and were seronegative at the last blood sampling on April 23, 2013 (Figure). The mean length of time between birth and the first detection of seronegative status was 180 days (range 120-240).

There are few reports regarding the decay of maternal antibodies against orthobunyaviruses in ruminants. Tsutsui and colleagues (*9*) showed that dairy calves lost their maternally derived antibodies against Akabane virus at ≈4 months of age, and Grimstad and colleagues (*10*) showed that young white-tailed deer lost their maternally derived antibodies against Jamestown Canyon virus at 5–6 months of age. Consistent with those findings, our results show that calves lose maternally derived SBV antibodies at ≈6 months of age and can then be effectively vaccinated against SBV.

Armin R.W. Elbers, Norbert Stockhofe, and Wim H.M. van der Poel

Author affiliation: Central Veterinary Institute, Lelystad, the Netherlands

DOI: http://dx.doi.org/10.3201/eid2005.130763

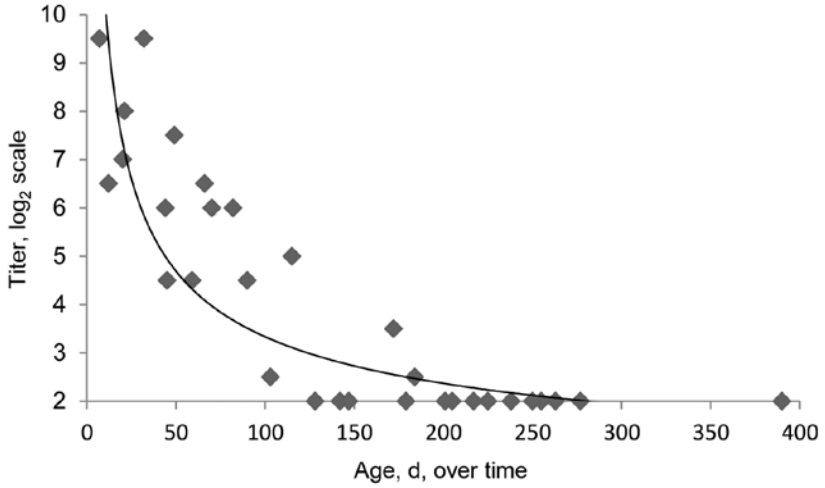


Figure. Schmallenberg virus antibody titers for 13 calves in a study to determine the decline of maternal antibodies in calves, the Netherlands, 2012–2013. Titers were determined by using a virus neutralization test and 2–4 blood samples per calf over time.

References

1. Yilmaz H, Hoffmann B, Turan N, Cizmecigil UY, Satir E, Richt JA, et al. Detection and partial sequencing of Schmallenberg virus in cattle and sheep in Turkey. Vector Borne Zoonotic Dis. Epub 2014 Feb 27.
2. European Food Safety Authority. Schmallenberg virus: analysis of the epidemiological data. (May 2013). Supporting publications 2013:EN-429 [cited 2013 May 20]. http://www.efsa.europa.eu/en/supporting/pub/429e.htm
3. Case P. SBV vaccine approval makes good progress [cited 2013 May 18]. Farmers Weekly. 2013 Feb 20. http://www.fwi.co.uk/articles/20/02/2013/137748/sbv-vaccine-approval-makes-good-progress.htm
4. Eschbaumer M, Eschweiler J, Hoffmann B. Long-term persistence of neutralising antibodies against bluetongue virus serotype 8 in naturally infected cattle. Vaccine. 2012;30:7142–3. http://dx.doi.org/10.1016/j.vaccine.2012.08.030
5. Kimman TG, Westenbrink F, Schreuder BEC, Straver PJ. Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. J Clin Microbiol. 1987; 25:1097–106.
6. Elbers ARW, Meiswinkel R, van Weezep E, Sloet van Oldruitenborgh-Oosterbaan MM, Kooi EA. Schmallenberg virus detected by RT-PCR in *Culicoides* biting midges captured during the 2011 epidemic in the Netherlands. Emerg Infect Dis. 2013;19:106–9. http://dx.doi.org/10.3201/eid1901.121054
7. Elbers ARW, Meiswinkel R, van Weezep E, Kooi EA, van der Poel WHM. Schmallenberg virus in *Culicoides* biting midges in the Netherlands in 2012. Transbound Emerg Dis. Epub 2013 Jul 24.
8. Loeffen W, Quak S, de Boer-Luijtze E, Hulst M, van der Poel WHM, Bouwstra R, et al. Development of a virus neutralisation test to detect antibodies against Schmallenberg virus and serological results in suspect and infected herds. Acta Vet Scand. 2012;54:44. http://dx.doi.org/10.1186/1751-0147-54-44
9. Tsutsui T, Yamamoto T, Hayama Y, Akiba Y, Nishiguchi A, Kobayashi S, et al. Duration of maternally derived antibodies against Akabane virus in calves: survival analysis. J Vet Med Sci. 2009;71:913–8. http://dx.doi.org/10.1292/jvms.71.913
10. Grimstad PR, Williams DG, Schmitt SM. Infection of white-tailed deer (*Odocoileus virginianus*) in Michigan with Jamestown Canyan virus (California serogroup) and the importance of maternal antibody in viral maintenance. J Wildl Dis. 1987;23:12–22. http://dx.doi.org/10.7589/0090-3558-23.1.12

Address for correspondence: Armin Elbers, Department of Epidemiology, Crisis Organization and Diagnostics, Central Veterinary Institute, Houtribweg 39, 8221 RA Lelystad, the Netherlands; email: armin.elbers@wur.nl

Linezolid-Resistant *Staphylococcus epidermidis*, Portugal, 2012

To the Editor: Linezolid is a therapeutic option for skin and soft tissue infections and pneumonia caused by multidrug-resistant gram-positive bacteria (e.g., *Staphylococcus* spp.), which occur at higher rates in Portugal than in other European countries (www.ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report-2013.pdf). *Staphylococcus epidermidis* are skin and mucosal commensal bacteria; infections in humans are mostly linked to indwelling medical devices. The ability of *S. epidermidis* to acquire resistance to antimicrobial drugs and to produce biofilm can seriously compromise the success of therapy; in many institutions worldwide, rates of methicillin resistance are >70% (*1*). Rates of *S. epidermidis* linezolid resistance on various continents have been low and are associated with mutations in the central loop of 23S rRNA V domain or ribosomal proteins (L3, L4, and L22) and with acquisition of the *cfr* gene, which codifies for ribosomal methyltransferase (*1–3*).

To our knowledge, in Portugal only 1 linezolid-resistant *S. epidermidis* isolate, from a dog with severe otitis, has been described (*4*). We report nosocomial emergence of methicillin- and linezolid-resistant *S. epidermidis* in Portugal.

We characterized 5 linezolid-resistant *Staphylococcus* isolates recovered during May–November 2012 from blood and catheters of patients in 4 wards of a 362-bed hospital in central Portugal. The origin of 1 isolate is unknown. Epidemiologic features are described in the Table. The patients had received linezolid during the present (n = 2 patients) or previous (n = 2 patients) hospitalizations, suggesting that the latter 2 patients could have been colonized with linezolid-resistant strains when discharged from the first hospitalization. Information about receipt of linezolid was not available for 1 patient.

S. epidermidis was identified by using a Vitek II system (bioMérieux, Marcy L'Étoile, France), and susceptibility to antimicrobial drugs was studied by using agar dilution (linezolid, vancomycin) or disk diffusion (another 10 drugs; Table) (*5*). All isolates were screened by PCR and sequenced for the *cfr* gene and for mutations in the 23S rRNA V domain and in genes (*rplC, rplD* and *rplV*) encoding the L3, L4, and L22 ribosomal proteins (*6–8*). Clonal relatedness was determined by pulsed-field gel electrophoresis (macrorestriction with *Sma*I) and by multilocus sequence typing (www.cdc.gov/hai/pdfs/labsettings/ar_mras_pfge_s_aureus.pdf and http://sepidermidis.mlst.net). *S. epidermidis* from patient 1 was searched for in vitro adherence to abiotic surfaces by using a biomass quantification assay (*9*) and strain ICE9 as a positive control.

All *S. epidermidis* isolates were resistant to multiple drugs, including linezolid (MIC>32 mg/L), cefoxitin, chloramphenicol, cotrimoxazole, ciprofloxacin, clindamycin, and aminoglycosides, and susceptible to only 4 drugs tested, including vancomycin (MIC = 2 mg/L) (Table). To characterize linezolid resistance, we compared the study isolates with linezolid-susceptible *S. epidermidis* RP62A/American Type Culture Collection 35984 sequence (GenBank accession no. CP000029). Study isolates contained the mutations T2530A, T2504A, and G2631T, although T2504A and G2631T were also present in linezolid-susceptible *S. epidermidis* RP62A (*1*,*2*). The most commonly reported G2576T mutation was not detected (*1*,*2*). We also compared study isolates with *S. epidermidis* RP62A and observed nucleotide mutations consistent with L94V (L101V from *S. epidermidis* American Type Culture Collection 12228, not associated with linezolid resistance) and G152D amino acid changes (*2*,*8*) and amino acid changes in the new D159E and A160P in L3 ribosomal protein. Mutations in this protein were linked to linezolid resistance, although definitive conclusions are not available (*8*). The *cfr* gene and mutations in ribosomal proteins L4 or L22 were not detected in the study isolates.

All isolates recovered had the same pulsed-field gel electrophoresis type and belonged to sequence type (ST) 2/clonal complex (CC) 5 (ST2 formerly belonged to CC2) (*10*) also detected among linezolid-resistant *S. epidermidis* from Europe, Brazil, and the United States (*1–3*). *S. epidermidis* from patient 1, considered representative of the observed clone, revealed a high ability to adhere to abiotic surfaces and grow in the biofilm form, which can facilitate infections associated with indwelling medical devices. This strain was classified as strongly adherent and had higher optical density (OD_{570nm} = 2.33 ± 0.34) than a blank sample (culture medium: Luria Bertani broth + glucose; OD_{570nm} = 0.2 ± 0.03). The OD_{570nm} of the positive-control was 2.69 ± 0.44.

S. epidermidis ST2/CC5 is disseminated in hospital settings worldwide and is characterized by a high level of genetic diversity, an increased recombination/mutation rate, biofilm production ability, and acquisition of a high number of staphylococcal cassette chromosome *mec* elements (*10*). In Portugal, *S. epidermidis* ST2/CC5 has been observed in the community (*10*). We report emergence of methicillin- and linezolid-resistant *S. epidermidis* in a hospital in Portugal and its persistence for at least 7 months. Identification of the successful multidrug-resistant *S. epidermidis* ST2/CC5 clonal lineage highlights the need for strict infection

Table. Epidemiologic features and antimicrobial drug resistance of linezolid-resistant *Staphylococcus epidermidis* isolates from a hospital, Portugal, 2012*

Characteristic	Patient no.†				
	1	2	3	4	5
Epidemiologic features					
Date of isolation	2012 May 8	2012 Aug 7	2012 Oct 23	2012 Nov 7	2012 Nov 11
Hospital ward	Men's surgery	Unknown	Medicine I	Emergency unit‡	Emergency unit‡
Pathology	Gastric neoplasia§¶	Unknown	Multiple§	Acute lung edema	Multiple
Clinical sample	Catheter	Blood	Catheter	Blood	Blood
Patient sex/age, y	M/75	Unknown	F/87	M/78	M/87
Previous linezolid	Yes	Unknown	Yes#	Yes‡	Yes‡
PFGE type	A	A	A	A	A
Sequence type	2**				
Biofilm production (OD_{570nm})	Strong (2.33 ± 0.34)**††				
Drug resistance					
Linezolid (MIC, mg/L)	R (32)	R (32)	R (32)	R (32)	R (32)
Vancomycin (MIC, mg/L)	S (2)	S (2)	S (2)	S (2)	S (2)
Cefoxitin	R	R	R	R	R
Gentamicin	R	R	R	R	R
Tobramycin	R	R	R	R	R
Ciprofloxacin	R	R	R	R	R
Clindamycin	R	R	R	R	R
Erythromycin	S	I	S	I	S
Quinupristin–dalfopristin	S	S	S	S	S
Chloramphenicol	R	R	R	R	R
Tetracycline	S	S	S	S	S
Cotrimoxazole	R	R	R	R	R
Molecular features					
cfr gene	–	–	–	–	–
23S rRNA mutations					
T2504A	+	+	+	+	+
G2631T	+	+	+	+	+
T2530A	+	+	+	+	+
L3 ribosomal protein mutations					
Leu94Val	+**				
Gly152Asp	+**				
Asp159Glu	+**				
Ala160Pro	+**				
L4 or L22 ribosomal protein mutations	None				

*PFGE, pulsed-field gel electrophoresis; OD, optical density; R, resistant; S, susceptible; I, Intermediate resistance; –, negative; +, positive. Blank cells indicate not tested.
†A sixth linezolid-resistant *S. epidermidis* isolate was detected in December 2012; however, access to this isolate was not possible during this study.
‡Patients 4 and 5 were hospitalized in Medicine II a month before linezolid-resistant *S. epidermidis* was isolated. Therapy with linezolid was started during this first hospitalization. For patient 4, duration of linezolid therapy was at least 12 d. For patient 5, duration of therapy is unknown.
§Long-stay hospitalization.
¶Followed up in oncology ward since 2011.
#Patient 3 received linezolid for 11 d before linezolid-resistant *S. epidermidis* was detected.
**Studied in S. *epidermidis* from patient 1 only, representative isolate of the PFGE type A.
††For the interpretation of the results, the cutoff optical density (ODc) was defined as 3 SDs above the mean OD of the negative control (culture medium). Strains were classified as nonadherent (OD≤ODc), weakly adherent (ODc<OD≤2×ODc), moderately adherent (2xODc<OD≤4×ODc), or strongly adherent (4×ODc<OD).

control procedures and revision of therapeutic strategies (e.g., linezolid use for the treatment of methicillin-resistant *Staphylococcus* spp. only when vancomycin is not a treatment option because of elevated MIC or clinical failures) to preserve therapeutic effectiveness of linezolid. Effective control of linezolid-resistant *S. epidermidis*, including among hospital-discharged patients who had received linezolid, is critical for preventing the potential for an epidemic in this hospital, and, on a larger scale, in Portugal, as has occurred for other gram-positive methicillin-resistant *S. aureus* and vancomycin-resistant enterococci.

Acknowledgments

We thank Conceição Faria, Sofia Almeida, and other members of the Serviço de Patologia Clínica do Centro Hospitalar da Cova da Beira (Covilhã, Portugal), who kindly provided the linezolid-resistant *S. epidermidis*.

This project was funded by a research grant from the Fundação para a Ciência e Tecnologia (PEst-C/EQB/LA0006/2011). R.B. is supported by a PhD fellowship of the Fundação para a Ciência e Tecnologia (grant no. SFRH/BD/61410/ 2009).

Mariana Barros, Raquel Branquinho, Filipa Grosso, Luísa Peixe, and Carla Novais

Author affiliaton: Faculdade de Farmácia da Universidade do Porto, Porto, Portugal

DOI: http://dx.doi.org/10.3201/eid2005.130783

References

1. Mendes RE, Deshpande LM, Costello AJ, Farrell DJ. Molecular epidemiology of *Staphylococcus epidermidis* clinical isolates from U.S. hospitals. Antimicrob Agents Chemother. 2012;56:4656–61. http://dx.doi.org/10.1128/AAC.00279-12
2. de Almeida LM, Lincopan N, Araújo MR, Mamizuka EM. Dissemination of the linezolid-resistant *Staphylococcus epidermidis* clone ST2 exhibiting the G2576T mutation in the 23S rRNA gene in a tertiary-care hospital, Brazil. J Antimicrob Chemother. 2012;67:768–9. http://dx.doi.org/10.1093/jac/dkr538
3. Liakopoulos A, Spiliopoulou I, Damani A, Kanellopoulou M, Schoina S, Papafragas E, et al. Dissemination of two international linezolid-resistant *Staphylococcus epidermidis* clones in Greek hospitals. J Antimicrob Chemother. 2010;65:1070–1. http://dx.doi.org/10.1093/jac/dkq065
4. Seixas R, Monteiro V, Carneiro C, Vilela CL, Oliveira M. First report of a linezolid-resistant MRSA (methicillin resistant *Staphylococcus aureus*) isolated from a dog with a severe bilateral otitis in Portugal. Revista Veterinaria. 2011;22:81–4.
5. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: twentieth informational supplement M100–S21. Wayne (PA): The Institute; 2010.
6. Kehrenberg C, Schwarz S. Distribution of florfenicol resistance genes *fex*A and *cfr* among chloramphenicol-resistant S*taphylococcus* isolates. Antimicrob Agents Chemother. 2006;50:1156–63. http://dx.doi.org/10.1128/AAC.50.4.1156-1163.2006
7. Toh SM, Xiong L, Arias CA, Villegas MV, Lolans K, Quinn J, et al. Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant *Staphylococcus aureus* resistant to the synthetic antibiotic linezolid. Mol Microbiol. 2007;64:1506–14. http://dx.doi.org/10.1111/j.1365-2958.2007.05744.x
8. Locke JB, Hilgers M, Shaw KJ. Novel ribosomal mutations in *Staphylococcus aureus* strains identified through selection with the oxazolidinones linezolid and torezolid (TR-700). Antimicrob Agents Chemother. 2009;53:5265–74. http://dx.doi.org/10.1128/AAC.00871-09
9. Novais A, Pires J, Ferreira H, Costa L, Montenegro C, Vuotto C, et al. Characterization of globally spread *Escherichia coli* ST131 isolates (1991 to 2010). Antimicrob Agents Chemother. 2012;56:3973–6. http://dx.doi.org/10.1128/AAC.00475-12
10. Rolo J, Lencastre H, Miragaia M. Strategies of adaptation of *Staphylococcus epidermidis* to hospital and community: amplification and diversification of *SCC*mec. J Antimicrob Chemother. 2012;67:1333–41. http://dx.doi.org/10.1093/jac/dks068

Address for correspondence: Carla Novais, Laboratório de Microbiologia. Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira nº 228, 4050-313 Porto, Portugal; email: casilva@ff.up.pt

Composite SCC*mec* Element in Single-locus Variant (ST217) of Epidemic MRSA-15 Clone

To the Editor: Since early epidemiologic studies of methicillin-resistant *Staphylococcus aureus* (MRSA) were published, it has been clear that the majority of nosocomial MRSA infections worldwide are caused by isolates derived from a few highly epidemic MRSA (EMRSA) clones. These are thought to have emerged through acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*) element by successful methicillin-susceptible *S. aureus* strains, within 5 major lineages or clonal complexes (CCs) including CC22 (*1*). Although epidemic clones are found worldwide, shifts of the predominant clones over time in which the emerging and usually more antibacterial drug–susceptible clones replace the older ones have been noted in countries, in small regions within countries, and in single hospitals (*2*). The reasons and mechanisms of such replacement as well as the epidemiologic dynamics leading to the success of a particular epidemic clone are largely unknown.

In Italy, isolations of classical EMRSA clones such as ST8-MRSA-I, ST247-MRSA-I, and ST239-MRSA-III decreased from the 1990s to the 2000s; during the same period ST228-MRSA-I increased, became established, and turned into the predominant clone in Italy (*3*). The genesis of other clones, such as ST8-MRSA-IV and ST22-MRSA-IV, which were associated with a tendency towards decreased multidrug resistance, was documented during 2000–2007 (*3*). Similar to occurrences in other European countries, the gentamicin-susceptible Panton-Valentine leukocidin–negative ST22-MRSA-IV clone, also known as EMRSA-15 (*1*), is now becoming predominant in Italy, replacing ST228-MRSA-I in hospital settings (*4*).

As part of another investigation, we recently isolated a MRSA strain from the nasal swab samples of a 5-year-old boy and his parents. The 3 isolates shared the same antibacterial drug resistance pattern (oxacillin and ciprofloxacin resistance) and proved to be identical by pulsed-field gel electrophoresis, SCC*mec* typing, and *agr* typing. Remarkably, ≈2 months earlier, the child had been admitted to a pediatric hospital for 10 days to be evaluated and treated for behavioral problems. A MRSA isolate, which was identified in a nasal sample obtained and analyzed just before discharge in the absence of clinical symptoms and was not further investigated, showed the same antibacterial drug resistance pattern as the 3 isolates collected later. In the absence of an epidemiologic history of exposure outside the hospital, it seems reasonable to assume that the strain was acquired by the child in the hospital and then transmitted to his parents.

Investigation of the genetic background of the strain isolated from the child's specimen, designated as Lu1, led to its assignment as ST217, a single-locus variant of EMRSA-15 within the same CC, CC22 (http://saureus.mlst.net), *agr* group I, and *spa* type t965. At times associated with ST22-MRSA-IV strains, t965 is a single repeat variant of t032, which is the most prevalent *spa* type of EMRSA-15; t965 and closely related *spa* types have been reported mainly in Germany and the United Kingdom (http://spa.ridom.de). Strain Lu1 was Panton-Valentine leukocidin–negative and lacked the ACME (arginine catabolic mobile element) cluster. By using current criteria (*5*), the SCC*mec* element was assigned to type IV(2B), which is consistent with the combination of a class B *mec* complex and a *ccrA2B2* (type 2) *ccr* complex. However, an additional *ccr* locus (*ccrC*, type 5) was found in the J3 region between *orfX* and IS*431*. The SCC*mec* element was thus identified as a composite type IV(2B&5). Sequencing of the *ccrC*-IS*431* segment (3,217 bp, GenBank accession no. HG315670), followed by performing analysis by using BLAST (http://blast.ncbi.nlm.nih.gov), displayed variable alignment scores and high-level nucleotide identities to the corresponding regions found downstream of *ccrC* in the SCC*mec* elements of MRSA reference strains of types III(3A), IV(2B&5), V(5C2&5), and VII(5C1) (*5*). The highest identity (3,216/3,217 nt) was with JCSC6944 (GenBank accession no. AB505629), an unspecified animal isolate of type V(5C2&5) from Japan, subtype c, belonging to the livestock-associated MRSA clone CC398 (*6*).

Very few ST217 strains, and none from Italy, are currently found in the MLST database (http://saureus.mlst.net), and data on such strains are scant in the literature. In particular, ST217-MRSA-IV was 1 of the dominant MRSA lineages isolated from patients in a hospital in Switzerland (*7*) and was detected in food samples of animal origin in Spain (*8*).

The composite SCC*mec* organization we detected in strain Lu1, featuring 2 *ccr* complexes (type 2 and 5), is similar to that described in 2 isolates that belong to different genetic lineages: 1 (ST100, CC5), later designated ZH47 (*5*,*9*), was identified in a sample from an inpatient in Switzerland (*7*); and the other (ST59, CC59, community-associated MRSA) from a pediatric patient in Taiwan (*10*).

Strain Lu1 (ST217, a single-locus variant of EMRSA-15) might have evolved from the ST22-MRSA-IV clone, which has recently been identified in hospitals in Italy (*3*,*4*). Its SCC*mec* organization may result from recombination events in which a type IV(2B) element acquired the *ccrC*-containing region downstream of *orfX* from SCC*mec* elements that normally contain it (*5*). However, a genetic exchange involving MRSA strains of animal origin cannot be excluded, considering the virtually identical sequence of the *ccrC*-IS*431* segment shared by strains Lu1 and JCSC6944, the latter being a CC398 LA-MRSA (*6*), and the isolation of ST217 strains from food samples of animal origin (*8*).

This work was supported in part by a grant from the Italian Ministry of Health to P.E.V.

Carla Vignaroli, Alessio Mancini, and Pietro E. Varaldo

Author affiliation: Università Politecnica delle Marche, Ancona, Italy

DOI: http://dx.doi.org/10.3201/eid2005.130934

References

1. Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc Natl Acad Sci U S A. 2002;99:7687–92. http://dx.doi.org/10.1073/pnas.122108599
2. Deurenberg RH, Stobberingh EE. The evolution of *Staphylococcus aureus*. Infect Genet Evol. 2008;8:747–63. http://dx.doi.org/10.1016/j.meegid.2008.07.007
3. Campanile F, Bongiorno D, Borbone S, Stefani S. Hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) in Italy. Ann Clin Microbiol Antimicrob. 2009;8:22. http://dx.doi.org/10.1186/1476-0711-8-22
4. Baldan R, Testa F, Lorè NI, Bragonzi A, Cichero P, Ossi C, et al. Factors contributing to epidemic MRSA clones replacement in a hospital setting. PLoS ONE. 2012;7:e43153. http://dx.doi.org/10.1371/journal.pone.0043153
5. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother. 2009;53:4961–7. http://dx.doi.org/10.1128/AAC.00579-09
6. Li S, Skov RL, Han X, Larsen AR, Larsen J, Sørum M, et al. Novel types of staphylococcal cassette chromosome *mec* elements identified in clonal complex 398 methicillin-resistant *Staphylococcus aureus* strains. Antimicrob Agents Chemother. 2011;55:3046–50. http://dx.doi.org/10.1128/AAC.01475-10
7. Qi W, Ender M, O'Brien F, Imhof A, Ruef C, McCallum N, et al. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Zurich, Switzerland (2003): prevalence of type IV SCC*mec* and a new SCC*mec* element associated with isolates from intravenous drug users. J Clin Microbiol. 2005;43:5164–70. http://dx.doi.org/10.1128/JCM.43.10.5164-5170.2005
8. Lozano C, López M, Gómez-Sanz E, Ruiz-Larrea F, Torres C, Zarazaga M. Detection of methicillin-resistant *Staphylococcus aureus* ST398 in food samples of animal origin in Spain. J Antimicrob Chemother. 2009;64:1325–6. http://dx.doi.org/10.1093/jac/dkp378
9. Heusser R, Ender M, Berger-Bächi B, McCallum N. Mosaic staphylococcal cassette chromosome *mec* containing two recombinase loci and a new *mec* complex, B2. Antimicrob Agents Chemother. 2007;51:390–3. http://dx.doi.org/10.1128/AAC.00921-06
10. Boyle-Vavra S, Ereshefsky B, Wang CC, Daum RS. Successful multiresistant community-associated methicillin-resistant *Staphylococcus aureus* lineage from Taipei, Taiwan, that carries either the novel staphylococcal chromosome cassette *mec* (SCC*mec*) type V_T or SCC*mec* type IV. J Clin Microbiol. 2005;43:4719–30. http://dx.doi.org/10.1128/JCM.43.9.4719-4730.2005

Address for correspondence: Pietro E. Varaldo, Unità di Microbiologia, Dipartimento di Scienze Biomediche e Sanità Pubblica, Università Politecnica delle Marche, Via Tronto 10/A, 60126 Ancona, Italy; email: pe.varaldo@univpm.it

Bartonella quintana in Body Lice from Scalp Hair of Homeless Persons, France

To the Editor: *Bartonella quintana* is a body louse–borne human pathogen that can cause trench fever, bacillary angiomatosis, endocarditis, chronic bacteremia, and chronic lymphadenopathy (*1*). Recently, *B. quintana* DNA was detected in lice collected from the heads of poor and homeless persons from the United States, Nepal, Senegal, Ethiopia, and the Democratic Republic of the Congo and in nits in France (*2*,*3*). The head louse, *Pediculus humanus capitis*, and the body louse, *Pediculus humanus humanus,* are obligatory ectoparasites that feed exclusively on human blood (*4*). Outside of their habitats, the 2 ecotypes are morphologically indistinguishable (*1*). Sequence variation in the PHUM540560 gene discriminates between head and body lice by determining the genotype of the lice (*5*). While surveying for trench fever among homeless persons in shelters in Marseille, France during October 2012–March 2013, we investigated the presence of *B. quintana* DNA in nits, larvae, and adult lice collected from mono-infested and dually infested persons and determined the genotypes of the specimens.

The persons included in this study received long-lasting insecticide-treated underwear; lice were collected by removing them from clothing, including underwear, pants, and shirts. Because body lice reside in the clothing of infested persons except when feeding, they are sometimes called clothing lice.

A total of 989 specimens were tested, including 149 (83 from clothing and 66 from hair) first–instar larvae hatched in the laboratory from eggs collected from 7 dually infested persons, and 840 adult body lice collected from the clothing of 80 mono-infested patients. We included DNA isolated from 3 nits collected from the hair of a mono-infested person who had previously been confirmed as positive for *B. quintana 6*) (Table).

Total DNA was extracted by using an EZ1 automated extractor (QIAGEN, Courtaboeuf, France) and subjected twice to real-time PCR specific for *B. quintana*. The first PCR targeted the 16S-23S intergenic spacer region. Positive samples were confirmed by using a second real-time PCR targeting the *yopP* gene (*6*). Samples that tested positive for *B. quintana* DNA were analyzed by multiplex real-time PCR that targeted the PHUM540560 gene (*5*). We used head and body lice that had known genotypes positive controls. Negative controls were included in each assay.

Of the hatched larvae, 5 (6%) of the 83 recovered from clothing and 7 (11%) of 66 from the hair (Table) of 4 of the 7 dually infested persons were positive for *B. quintana* DNA (online Technical Appendix Table 1 wwwnc.cdc.gov/EID/article/20/5/13-1242-Techapp1.pdf). Of the 840 adult body lice, 174 (21%) collected from 42 (53%) of 80 of the mono-infested persons contained *B. quintana* DNA (Table, online Technical Appendix 2). The multiplex real-time PCR that targeted the PHUM540560 gene clearly identified all nits, larvae, and adult lice as belonging to the body lice lineage. Negative controls remained negative in all PCR-based experiments.

For 2 decades, *B. quintana* DNA has been regularly detected in lice collected from the heads of persons living in poverty, but it had not been detected in head lice that infest schoolchildren (*7*,*8*). All of the lice collected during this study that tested positive for *B. quintana* from homeless persons were body lice, including some that were recovered from hair. This observation supports our assertion that body lice are not confined to the body. The 3 eggs that were removed from the hair of a mono-infested homeless person whose samples tested positive for *B. quintana* were also body lice. During the clinical examination, no adult head lice or adult body lice were found on that person, confirming that the patient had been heavily infested with body lice in the past, not head lice. The nits were most likely laid by body lice that migrated toward the patient's head. When a member of

Table. Distribution of *Bartonella quintana* DNA in nits, larvae, and adult body lice collected from hair and clothing of homeless persons in shelters, Marseille, France, October 2012–March 2013*

Location	No. persons		No. (%) lice positive for *B. quintana* DNA	Reference
	Dually infested, n = 7	Monoinfested, n = 80		
Hair				
Nits	0	3	3 (100)	(*6*)
Hatched larvae	66	0	7 (10.60)	This study
Clothing				
Hatched larvae	83	0	5 (6.00)	This study
Adults	0	840	174 (20.70)	This study

*All lice were identified as body lice. Study participants were provided with long-lasting insecticide-treated underwear, and killed body lice were collected from the clothing of infested persons.

this research team (DR) collected the eggs from the hair shaft, they were found ≈3 3.5 cm from the hair follicle. Because hair grows ≈1.25 cm per month, the louse infestation occurred ≈3 months before egg collection (*6*).

Homeless persons that we have monitored for many years are often heavily infested by body lice but are also occasionally infested with head lice. Before genetic tools that differentiate the head and body louse lineages were available (*5*), it was speculated that body lice may have originated from head lice (*9*). From our study, it is clear that under conditions of massive infestation, body lice can migrate and colonize hair; the opposite may also be true. However, there is no evidence that body lice are capable of causing an outbreak of lice living on the head, as happens among schoolchildren that have been found to be infested only by head lice. This suggests that body lice cannot thrive in the environment of head lice, which infest millions of children worldwide (*10*), further suggesting that outbreaks of trench fever are most likely not linked to head lice in industrialized countries. In conclusion, by analyzing lice harvested from the heads and clothing of homeless persons, we have shown that the 2 ecotypes belong to the same body lice population.

The text has been edited by American Journal Experts under certificate verification key 51F8-8A17-1F51-90DD-705C.

Rezak Drali, Abdoul Karim Sangaré, Amina Boutellis, Emmanouil Angelakis, Aurélie Veracx, Cristina Socolovschi, Philippe Brouqui, and Didier Raoult

Author affiliations: Aix Marseille Université, Marseille, France; and Institut Hospitalo-Universitaire Méditerranée Infection, 13005, Marseille

DOI: http://dx.doi.org/10.3201/eid2005.131242

References

1. Brouqui P. Arthropod-borne diseases associated with political and social disorder. Annu Rev Entomol. 2011;56:357–74. http://dx.doi.org/10.1146/annurev-ento-120709-144739
2. Boutellis A, Mediannikov O, Bilcha KD, Ali J, Campelo D, Barker SC, et al. *Borrelia recurrentis* in head lice, Ethiopia. Emerg Infect Dis. 2013;19:796–8.
3. Piarroux R, Abedi AA, Shako JC, Kebela B, Karhemere S, Diatta G, et al. Plague epidemics and lice, Democratic Republic of the Congo. Emerg Infect Dis. 2013;19:505–6. http://dx.doi.org/10.3201/eid1903.121542
4. Gratz NG. Emerging and resurging vector-borne diseases. Annu Rev Entomol. 1999;44:51–75. http://dx.doi.org/10.1146/annurev.ento.44.1.51
5. Drali R, Boutellis A, Raoult D, Rolain JM, Brouqui P. Distinguishing body lice from head lice by multiplex real-time PCR analysis of the Phum_PHUM540560 gene. PLoS ONE. 2013;8:e58088. http://dx.doi.org/10.1371/journal.pone.0058088
6. Angelakis E, Rolain JM, Raoult D, Brouqui P. *Bartonella quintana* in head louse nits. FEMS Immunol Med Microbiol. 2011;62:244–6. http://dx.doi.org/10.1111/j.1574-695X.2011.00804.x
7. Fournier PE, Ndihokubwayo JB, Guidran J, Kelly PJ, Raoult D. Human pathogens in body and head lice. Emerg Infect Dis. 2002;8:1515–8. http://dx.doi.org/10.3201/eid0812.020111
8. Bouvresse S, Socolovshi C, Berdjane Z, Durand R, Izri A, Raoult D, et al. No evidence of *Bartonella quintana* but detection of *Acinetobacter baumannii* in head lice from elementary schoolchildren in Paris. Comp Immunol Microbiol Infect Dis. 2011;34:475–7. http://dx.doi.org/10.1016/j.cimid.2011.08.007
9. Li W, Ortiz G, Fournier PE, Gimenez G, Reed DL, Pittendrigh B, et al. Genotyping of human lice suggests multiple emergencies of body lice from local head louse populations. PLoS Negl Trop Dis. 2010;4:e641. http://dx.doi.org/10.1371/journal.pntd.0000641
10. Chosidow O, Chastang C, Brue C, Bouvet E, Izri M, Monteny N, et al. Controlled study of malathion and *d*-phenothrin lotions for *Pediculus humanus* var *capitis*-infested schoolchildren. Lancet. 1994;344:1724–7. http://dx.doi.org/10.1016/S0140-6736(94)92884-3

Address for correspondence: Didier Raoult, Institut Hospitalo-Universitaire Méditerranée Infection, 27 boulevard Jean Moulin, 13385 Marseille CEDEX 5, France; email: didier.raoult@gmail.com

Myasthenia Gravis Associated with Acute Hepatitis E Infection in Immunocompetent Woman

To the Editor: Hepatitis E virus (HEV) is a common cause of acute hepatitis in developing countries. The course of acute hepatitis E is usually benign, except in pregnant women and in immunocompromised patients, who are prone to a lethal or chronic outcome of the disease. Since 2001, hepatitis E has been emerging in industrialized countries, and neurologic manifestations such as Guillain-Barré syndrome, brachial neuritis, transverse myelitis, and cranial nerve palsies have been reported in patients with acute or chronic forms of the disease (*1*–*6*). Most cases with neurologic manifestations have been characterized by infection with genotype 3 HEV. Data are not available to indicate whether this association between HEV infection and neurologic manifestations is related to a specific antigenic stimulus provided by HEV or is linked to the more comprehensive assessment for such neurologic conditions that is available in industrialized countries or to a reporting bias. We report a case of HEV infection in an immunocompetent woman who had muscle-specific kinase (MuSK) antibody–positive myasthenia gravis associated with HEV replication.

A 33-year-old woman was hospitalized in France for subacute asthenia and intermittent symptoms including dysarthria, dysphagia, muscle weakness, and diplopia. She had no family history of autoimmune disease and no notable personal medical history; she had not received any recent vaccinations and had not traveled outside France during the previous year. Physical examination showed no pyramidal, vestibular, or

cerebellar syndromes, and all tendon reflexes were typical.

On admission, the patient's liver function tests showed elevated alanine transaminase (190 UI/L). Test results were within reference ranges for aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, and creatine kinase levels. Antibody tests were negative for hepatitis B virus, hepatitis C virus, HIV, human T-lymphotropic viruses 1 and 2, and *Treponema pallidum*, but testing for cytomegalovirus and Epstein-Barr virus showed previous exposure. Serum samples were positive for HEV IgM (index 11) and negative for HEV IgG (index 0.71) by Adaltis microplate ELISA (EIAgen; Adaltis, Casalecchio Di Reno, Italy). HEV RNA was detected in serum and fecal samples by using HEV reverse transcription PCR (RT-PCR) (Ceeram, La Chapelle sur Erdre, France) on the SmartCycler II instrument (Cepheid, Sunnyvale, CA, USA). Sequencing studies showed that the HEV strain belonged to genotype 3f.

Brain magnetic resonance imaging results were normal, and results for analysis of cerebrospinal fluid were normal (leukocytes <5 cells/μL, glucose 3.2 mmol/L, protein 38 mg/dL, HEV RT-PCR negative). A nerve conduction study, performed even though the patient was not symptomatic, did not find any abnormality. Pharmacologic testing with prostigmine (0.5 mg intravenously) did not result in symptom improvement. Computed tomography scan of the mediastinum showed no thymoma. Test results for anti-acetylcholine receptor antibodies were negative; however, results were positive for MuSK antibodies (18.7 U/mL by radioimmunoassay; RSR Ltd., Cardiff, Wales, UK). These data, combined with physical examination, confirmed the diagnosis of myasthenia gravis.

Given this association of myasthenia and acute HEV infection, we suspected the potent role of HEV infection in the neurologic symptoms. Therapy was started with ribavirin (1,000 mg/day for 1 month) and intravenous immunoglobulin (IVIG) (1 g/kg/d for 2 days). Alanine transaminase levels rapidly decreased, and after 3 months, test results for HEV IgM and HEV RT-PCR in serum were negative. IVIG treatment relieved symptoms in the short term, but symptoms returned 15 days after treatment ended, requiring continuation of IVIG every 4 weeks for 6 months. Four months after the last infusion of IVIG, the patient reported mild 4-limb fatigability after exercise, but results of objective neurologic examination were normal. Results for MuSK antibodies were still positive, and treatment with azathioprine (150 mg/d) was started. Three weeks later, the patient required another infusion of IVIG for difficulty in swallowing, dyspnea, and 4-limb weakness, but she was free of symptoms for the remaining 5 months of follow-up.

In conclusion, we describe a case of anti-MuSK myasthenia gravis associated with acute HEV in a young, immunocompetent patient in France. Because myasthenia gravis with MuSK antibodies is rare (≈10% of myasthenia gravis cases) (*7*), the potential role of HEV infection as a trigger of autoimmune disorders should be investigated. Some cases of anti-MuSK myasthenia gravis associated with HIV (*8*) or Epstein-Barr virus (*9*) infections have been reported. Nevertheless, our findings do not enable us to draw conclusions regarding causality. Our observation might suggest a coincidental temporal association between HEV infection and myasthenia gravis or a triggering of autoimmunity by HEV. Moreover, anti-MuSK myasthenia gravis is usually characterized by a rapidly progressive course with moderate to severe symptoms (*7*). The initial unusually benign clinical course in this patient might be explained by the effect of early ribavirin treatment or, more likely, by a particular type of MuSK antibodies (*10*).

Aude Belbezier, Alban Deroux, Françoise Sarrot-Reynauld, Sylvie Larrat, and Laurence Bouillet

Author affiliations: Centre Hospitalier Universitaire de Grenoble, Grenoble, France (A. Belbezier, A. Deroux, F. Sarrot-Reynauld, S. Larrat, L. Bouillet); and Université Joseph Fourier Grenoble 1, Grenoble (F. Sarrot-Reynauld, S. Larrat, L. Bouillet)

DOI: http://dx.doi.org/10.3201/eid2005.131551

References

1. Cheung MC, Maguire J, Carey I, Wendon J, Agarwal K. Review of the neurological manifestations of hepatitis E infection. Ann Hepatol. 2012;11:618–22.
2. Peri AM, Milazzo L, Meroni L, Antinori S. Radiculoneuropathy associated with acute hepatitis E. Dig Liver Dis. 2013;45:963–4. http://dx.doi.org/10.1016/j.dld.2013.05.006
3. GeurtsvanKessel CH. Islam Z, Mohammad QD, Jacobs BC, Endtz HP, Osterhaus AD. Hepatitis E and Guillain-Barré syndrome. Clin Infect Dis. 2013;57:1369–70 . http://dx.doi.org/10.1093/cid/cit512
4. Scharn N, Ganzenmueller T, Wenzel JJ, Dengler R, Heim A, Wegner F. Guillain-Barré syndrome associated with autochthonous infection by hepatitis E virus subgenotype 3c. Infection. 2014;42:171–3. http://dx.doi.org/10.1007/s15010-013-0448-5
5. Despierres LA, Kaphan E, Attarian S, Cohen-Bacrie S, Pelletier J, Pouget J, et al. Neurologic disorders and hepatitis E, France, 2010. Emerg Infect Dis. 2011;17:1510–2 .
6. Kamar N, Bendall RP, Peron JM, Cintas P, Prudhomme L, Mansuy JM, et al. Hepatitis E virus and neurologic disorders. Emerg Infect Dis. 2011;17:173–9. http://dx.doi.org/10.3201/eid1702.100856
7. Evoli A, Padua L. Diagnosis and therapy of myasthenia gravis with antibodies to muscle-specific kinase. Autoimmun Rev. 2013;12:931–5. http://dx.doi.org/10.1016/j.autrev.2013.03.004
8. Kurokawa T, Nishiyama T, Yamamoto R, Kishida H, Hakii Y, Kuroiwa Y. Anti-MuSK antibody positive myasthenia gravis with HIV infection successfully treated with cyclosporin: a case report. Rinsho Shinkeigaku. 2008;48:666–9. http://dx.doi.org/10.5692/clinicalneurol.48.666
9. Bhibhatbhan A, Kline G, Vincent A, Toth C. Anti-MuSK myasthenia gravis presenting with Epstein-Barr virus–associated mononucleosis and immune-mediated diabetes mellitus. Muscle Nerve.

2007;36:264–6. http://dx.doi.org/10.1002/mus.20746
10. Zouvelou V, Stamboulis E, Skriapa L, Tzartos SJ. MuSK-Ab positive myasthenia: not always grave. J Neurol Sci. 2013;331:150–1. http://dx.doi.org/10.1016/j.jns.2013.05.001

Address for correspondence: Françoise Sarrot-Reynauld, Clinique Universitaire de Médecine Interne, Pôle Pluridisciplinaire de Médecine, Centre Hospitalier Universitaire de Grenoble, CS10217, 38043 Grenoble Cedex, France; email: FSarrot-Reynauld@chu-grenoble.fr

Ciprofloxacin-Resistant *Salmonella enterica* Serotype Kentucky Sequence Type 198

To the Editor: Mulvey et al. (*1*) reported the emergence of ciprofloxacin resistance in *Salmonella enterica* serovar Kentucky of multilocus sequence type 198 (ST198) in Canada (*1*). Ciprofloxacin resistance in *S. enterica* ser. Kentucky was reported in 2011 in patients from Europe, most of whom had traveled to Africa and the Middle East (*2*). Since then, *S. enterica* ser. Kentucky ST198 with additional resistance to third-generation cephalosporins and carbapenems has been reported from France and Morocco, again associated with travel (*3*). Poultry has been implicated as the most likely vehicle for infection by this sequence type (*2*,*3*). Resistance to third-generation cephalosporins and carbapenems has not been seen in North America; however, the emergence of ciprofloxacin-resistant infections has been observed (*1*).

In the United States, *S. enterica* ser. Kentucky is the most common serotype isolated from chickens and the second most common found among retail chicken, but ciprofloxacin resistance has not been documented among these sources (*4*). We sought to determine if ciprofloxacin- or ceftriaxone-resistant *S. enterica* ser. Kentucky has emerged in humans in the United States. We examined isolates and data from the National Antimicrobial Resistance Monitoring System to document antimicrobial resistance and sequence type and to assess possible risk factors for acquiring infection.

Participating state and local public health laboratories submit every 20th nontyphoidal *Salmonella* (NTS) isolate to the Centers for Disease Control and Prevention for susceptibility testing. MICs of ≥15 antimicrobial agents were determined by using broth microdilution (Sensititer, Cleveland, OH, USA) according to the manufacturer's instructions. Where available, Clinical and Laboratory Standards Institute performance standards were used for interpretation of MICs; otherwise, interpretations established by the National Antimicrobial Resistance Monitoring System were used (*5*,*6*).

During 2009–2012, a total of 21 (0.2%) of the 9,225 NTS isolates tested were *S. enterica* ser. Kentucky. Six (29%) were resistant to ciprofloxacin; all were susceptible to ceftriaxone (Table) (*5*). As was observed in Canada, the 6 resistant isolates were >80% similar by pulsed-field gel electrophoresis analysis (*Xba*I; data not shown), and all 6 were ST198. Although a rare cause of human infection, *S. enterica* ser. Kentucky represented 23% (6/26) of all ciprofloxacin-resistant NTS detected during 2009–2012.

The median age of the 6 patients with ciprofloxacin-resistant *S. enterica* ser. Kentucky infections was 32 years (range 9 months–56 years); 5 (83%) were female. Of the 4 patients for whom information was available, 2 were hospitalized, and 1 died. Specimen sources were stool (n = 3) and urine (n = 3). Travel histories were obtained for 5 patients, and all had traveled internationally in the 7 days before specimen submission: 2 were residents of other countries (Saudi Arabia and Ethiopia), and 3 were US residents who had returned from travel to India. By comparison, only 3 of 10 patients with ciprofloxacin-susceptible infections had traveled ($p = 0.02$).

Resistance to ciprofloxacin in *Salmonella* is a growing concern because it limits treatment options for invasive disease. We describe ciprofloxacin-resistant *S. enterica* ser. Kentucky isolated from 6 patients in the United States. The emerging global story of *S. enterica* ser. Kentucky ST198 demonstrates the need for international integration of surveillance for antimicrobial drug resistance.

Table. Patient and isolate information for 6 cases of infection with ciprofloxacin-resistant *Salmonella enterica* serotype Kentucky sequence type 198 detected by the National Antimicrobial Resistance Monitoring System, United States, 2009–2012*

Isolate ID	Patient age, y/sex	Patient race	Patient travel history	Year specimen collected	Specimen type	Antimicrobial resistance†
AM41047	<1/F	Black	Ethiopia	2009	Stool	AMP, FIS, GEN, NAL, STR, TET
AM45820	54/F	Unknown	Unknown	2010	Urine	AMP, COT, FIS, GEN, NAL, STR, TET
AM47052	56/F	Asian	India	2011	Urine	AMP, FIS, GEN, NAL, STR, TET
2012AM-1081	2/F	Asian	India	2012	Stool	AMP, NAL
AM50773	37/M	Asian	India	2012	Stool	AMP, AUG, FIS, GEN, NAL, STR, TET
2012AM-0353	42/F	White	Saudi Arabia	2012	Urine	AMP, FIS, FOX, KAN, NAL, STR, TET

*ID, identification; AMP, ampicillin; AUG, amoxicillin-clavulanic acid; COT, trimethoprim-sulfamethoxazole; FIS, sulfisoxazole; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline.
†Resistance of isolate from infected patient to antimicrobial agents other than ciprofloxacin.

Regan Rickert-Hartman and Jason P. Folster

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: http://dx.doi.org/10.3201/eid2005.131575

References

1. Mulvey MR, Boyd DA, Finley R, Fakharuddin K, Langner S, Allen V, et al. Ciprofloxacin-resistant *Salmonella enterica* serovar Kentucky in Canada. Emerg Infect Dis. 2013;19:999–1001. http://dx.doi.org/10.3201/eid1906.121351
2. Le Hello S, Hendriksen RS, Doublet B, Fisher I, Nielsen EM, Whichard JM, et al. International spread of an epidemic population of *Salmonella enterica* serotype Kentucky ST198 resistant to ciprofloxacin. J Infect Dis. 2011;204:675–84. http://dx.doi.org/10.1093/infdis/jir409
3. Le Hello S, Harrios D, Bouchrif B, Sontag L, Elhani D, Guibert V, et al. Highly drug-resistant *Salmonella enterica* serotype Kentucky ST198-X1: a microbiological study. Lancet Infect Dis. 2013;13:672–9. http://dx.doi.org/10.1016/S1473-3099(13)70124-5
4. US Food and Drug Administration. National Antimicrobial Resistance Monitoring System retail meat annual report, 2011 [cited 2013 Jul 1]. http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm334828.htm
5. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; Twenty-third informational supplement. CLSI document M100–S23. Wayne (PA): The Institute; 2013.
6. Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System human isolates annual report, 2011 [cited 2013 Jul 1]. http://www.cdc.gov/narms/pdf/2011-annual-report-narms-508c.pdf

Address for correspondence: Jason P. Folster, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G29, Atlanta, GA 30329, USA; email: gux8@cdc.gov

Unique Strain of Crimean–Congo Hemorrhagic Fever Virus, Mali

To the Editor: Crimean-Congo hemorrhagic fever (CCHF) is an acute viral infection that causes mild to severe hemorrhagic fever characterized by petechiae, ecchymosis, disseminated intravascular coagulation, and multi-organ failure (*1*). The etiologic agent, CCHF virus (CCHFV; family *Bunyaviridae,* genus *Nairovirus*), is maintained in enzootic cycles involving agricultural and wild animals and the vector, *Hyalomma* ticks. (*2*). CCHF predominantly affects persons who have 1) substantial contact with ticks and/or agricultural animals in areas where CCHF is endemic or 2) close contact with infected persons, predominantly close relatives and health care workers. The case-fatality rate for CCHF is generally accepted as 30% (*1*).

CCHF has a wide geographic distribution; cases have been reported in >30 countries across Africa, southeastern Europe, the Middle East, and western Asia. In the western African countries of Nigeria, Mauritania, and Senegal, serologic evidence of CCHFV infections in humans and agricultural animals has been documented frequently (*3*–*5*); however, reports of the disease in humans have been limited to Senegal and Mauritania (*6*,*7*). In neighboring Mali, where the tick vector is known to be present, little information exists regarding the presence of CCHFV. Thus, to determine if the virus is circulating undetected in Mali, we conducted a study to determine if CCHFV is present in *Hyalomma* ticks in the country.

In November 2011 and March 2012, unfed *Hyalomma* ticks (adults and nymphs) were collected from 20 cattle at the Daral livestock market (12° 49.855′ N, 08° 05.651′ W) near the town of Kati, Mali, ≈25 km from the capital, Bamako. In the field, ticks were visually identified to genus and pooled accordingly (3–4 ticks per pool, all collected from the same animal). A total of 23 tick pools, representing 80 ticks, were manually homogenized, and RNA was extracted and tested for the presence of CCHFV RNA by using in-house assays that selected for 3 virus genes. Of the 23 tick pools tested, 1 was positive for CCHFV by all 3 assays. Phylogenetic analysis of the complete nucleocapsid protein gene (KF793333) showed that the CCHFV strain from Mali most closely resembled a strain from Mauritania (GenBank accession no. ArD39554), sharing 98% sequence identity (Figure, panel A).

Further analysis of fragments of the medium segment (pre-Gn coding region, KF793334) and large segment (polymerase coding region, KF793335) confirmed these findings, showing sequence identities of 91% and 98%, respectively, with ArD39554 (Figure, panels B, C). In a Biosafety Level 4 facility at Rocky Mountain Laboratories, Hamilton, Montana, USA, the original homogenates from the positive pool were passaged in multiple cell lines. After 3 passages, no discernible cytopathic effect was observed and, aside from the initial passage, CCHFV RNA was not detected.

Genetic identification of ticks in the CCHFV RNA–positive pool was conducted as described (*8*,*9*). Amplified sequences most closely resembled those of *H. dromedarii*, (97.2%–100% sequence identity), although genetically, we cannot exclude the possibility that *H. truncatum* and *H. rufipes* were present with individual sequence identities of >97% to published sequences.

The Daral cattle market in Kati is the largest of its kind in Mali, and animals from across the country come into the market every week. Although the market provided a convenient opportunity for collecting ticks, we cannot determine where the infected

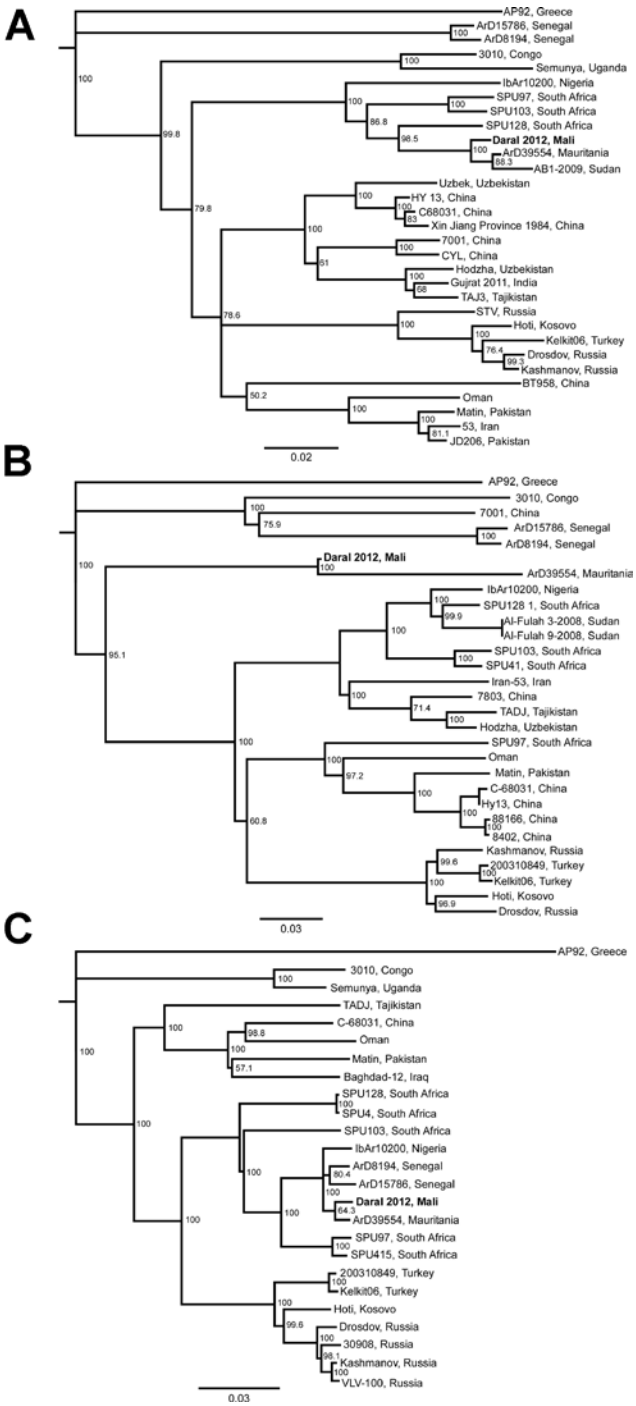


Figure. Phylogenetic analysis of Crimean–Congo hemorrhagic fever virus (CCHFV) was conducted on the complete nucleoprotein (small genomic segment, nt ≈50–1,500) (A), a 900-bp fragment of the glycoprotein precursor (medium genomic segment, nt ≈4190–5060) (B), and a 1,200-bp fragment of the viral polymerase (large genomic segment, nt ≈590–1760) (C). The fragments were amplified from pooled ticks, and sequence analysis was conducted by using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/). Trees were constructed by using the Jukes–Cantor neighbor-joining method with bootstrapping to 10,000 iterations and compared with published sequences of full-length small, medium, and large segments. Bold indicates CCHFV strain from *Hyalomma* ticks that were collected from cattle at the Daral livestock market near the town of Kati, Mali. Scale bars indicate substitutions per site.

ticks, and possibly cattle, contracted CCHFV because the animals traversed great distances on foot before arriving at the market. Nevertheless, this study demonstrates the presence of a distinct strain of CCHFV in *Hyalomma* ticks in Mali, thereby expanding the geographic distribution of this virus in western Africa. Not surprisingly, the highest sequence identity for the CCHFV strain from Mali is to strains known to circulate in neighboring countries (*10*). We propose Daral 2012 Mali as the temporary designation for this sequence. Unfortunately, our attempts to isolate the virus were unsuccessful, most likely because of processing and storage conditions for homogenates used in these studies.

Species of *Hyalomma* ticks are widely distributed across western Africa, and although reports of CCHF are limited to a few countries, CCHFV is most likely circulating undetected in vast areas of this region. No cases of CCHF have been reported in Mali; however, on the basis of our findings, the potential for human infections exists. Thus, CCHF should be considered in the differential diagnosis of febrile illnesses, with or without hemorrhagic symptoms, in residents of Mali and for persons with a recent history of travel to this country.

The ease of CCHFV transmission and the high case-fatality rate associated with infection could have a potentially substantial effect on public health. Future studies in Mali are required to define the geographic distribution of infected ticks and animals and to isolate CCHFV to help focus public health preparedness and countermeasures. In addition, across Mali, operational protocols should be reviewed for persons working at jobs in which the risk for CCHFV transmission is high (e.g., occupations with direct contact with agricultural animals and/or animal blood products), and appropriate countermeasures should be put in place to prevent transmission among such persons.

Acknowledgments

We are indebted to the chief of Daral for allowing us access to the Kati cattle market and to the individual ranchers for providing ticks from their cattle. In addition, we thank Seydou Doumbia, Sekou Traore, Richard Sakai, Joseph Shott, and Mark Pineda for logistics support; Robert J. Fischer, Brandi Williamson, Eric Dahlstrom, and Stephen Porcella for technical assistance; and Heather Murphy for help preparing the figures.

This work was funded by the International Centers for Excellence in Research program, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Marko Zivcec, Ousmane Maïga, Ashley Kelly, Friederike Feldmann, Nafomon Sogoba, Tom G. Schwan, Heinz Feldmann, and David Safronetz

Author affiliations: National Institutes of Health, Hamilton, Montana, USA (M. Zivcec, A. Kelly, F. Feldmann, T.G. Schwan, H. Feldmann, D. Safronetz); University of Manitoba, Winnipeg, Manitoba, Canada (M. Zivcec, H. Feldmann); and University of Sciences, Techniques and Technologies of Bamako, Bamako, Mali (O. Maïga, N. Sogoba)

DOI: http://dx.doi.org/10.3201/eid2005.131641

References

1. Ergonul O. Crimean–Congo hemorrhagic fever virus: new outbreaks, new discoveries. Curr Opin Virol. 2012;2:215–20. http://dx.doi.org/10.1016/j.coviro.2012.03.001
2. Whitehouse CA. Crimean–Congo hemorrhagic fever. Antiviral Res. 2004;64:145–60. http://dx.doi.org/10.1016/j.antiviral.2004.08.001
3. David-West TS, Cooke AR, David-West AS. Seroepidemiology of Congo virus (related to the virus of Crimean haemorrhagic fever) in Nigeria. Bull World Health Organ. 1974;51:543–6.
4. Gonzalez JP, LeGuenno B, Guillaud M, Wilson ML. A fatal case of Crimean–Congo haemorrhagic fever in Mauritania: virological and serological evidence suggesting epidemic transmission. Trans R Soc Trop Med Hyg. 1990;84:573–6. http://dx.doi.org/10.1016/0035-9203(90)90045-G
5. Wilson ML, LeGuenno B, Guillaud M, Desoutter D, Gonzalez JP, Camicas JL. Distribution of Crimean–Congo hemorrhagic fever viral antibody in Senegal: environmental and vectorial correlates. Am J Trop Med Hyg. 1990;43:557–66.
6. Nabeth P, Cheikh DO, Lo B, Faye O, Vall IO, Niang M, et al. Crimean–Congo hemorrhagic fever, Mauritania. Emerg Infect Dis. 2004;10:2143–9. http://dx.doi.org/10.3201/eid1012.040535
7. Tall A, Sall AA, Faye O, Diatta B, Sylla R, Faye J, et al. Two cases of Crimean–Congo haemorrhagic fever (CCHF) in two tourists in Senegal in 2004. Bull Soc Pathol Exot. 2009;102:159–61.
8. Rees DJ, Dioli M, Kirkendall LR. Molecules and morphology: evidence for cryptic hybridization in African *Hyalomma* (Acari: Ixodidae). Mol Phylogenet Evol. 2003;27:131–42. http://dx.doi.org/10.1016/S1055-7903(02)00374-3
9. Barker SC. Distinguishing species and populations of rhipicephaline ticks with its 2 ribosomal RNA. J Parasitol. 1998;84:887–92. http://dx.doi.org/10.2307/3284614
10. Deyde VM, Khristova ML, Rollin PE, Ksiazek TG, Nichol ST. Crimean–Congo hemorrhagic fever virus genomics and global diversity. J Virol. 2006;80:8834–42. http://dx.doi.org/10.1128/JVI.00752-06

Address for correspondence: David Safronetz, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 S 4th St, Hamilton, MT 59840, USA; email: safronetzd@niaid.nih.gov

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.

Coxsackievirus A16 Encephalitis during Obinutuzumab Therapy, Belgium, 2013

To the Editor: Enterovirus infections are associated with many clinical manifestations, and specific virus groups or serotypes are associated with specific manifestations. Coxsackievirus A16, a common cause of hand, foot and mouth disease, rarely causes encephalitis. Although most enterovirus infections are cleared by cellular immune responses, invasive enterovirus disease is prevented or controlled by neutralizing antibodies (*1*). Thus, patients with humoral immunodeficiencies are susceptible to serious enterovirus infections.

Nine cases of enteroviral encephalitis (1 caused by echovirus 13, 1 caused by coxsackievirus A16, 2 caused by enterovirus 71, and 5 caused by unknown enteroviruses) have been reported after therapy with rituximab, a monoclonal antibody (MAb) that causes secondary hypogammaglobulinemia (*2*). We describe coxsackievirus A16 encephalitis in a patient who was receiving treatment with the MAb obinutuzumab.

A 67-year-old woman with non-Hodgkin lymphoma showed complete remission after 6 cycles of treatment with bendamustine and obinutuzumab. Induction immunochemotherapy was followed by obinutuzumab maintenance therapy. At admission, she had received 7 of 12 scheduled treatments.

The patient was hospitalized because of a history of high-grade fever that did not respond to antimicrobial drugs, confusion, general weakness, and urinary incontinence. She had a neutrophil count of 3.1×10^9 cells/L but had severe lymphocytopenia (0.3×10^9 cells/L and an absolute CD4 cell count of 0.082×10^9 cells/L) and low

serum immunoglobulin levels (IgG 3.86 g/L [reference range 7.51–15.6 g/L], IgA 0.07 g/L [reference range 0.82–4.53 g/L], and IgM 0.13 g/L [reference range 0.46–3.04 g/L]). Cerebrospinal fluid (CSF) samples were collected on days 1, 4, and 6. CSF leukocyte counts increased from 14 cells/mm^3 (day 1) to 60 cells/mm^3 (day 4) (35% and 27% lymphocytes, respectively). Cytologic and immunophenotypic analyses showed no cerebromeningeal lymphoma infiltration. Total protein levels in CSF increased from 561 mg/L on day 1 to 771 mg/L on days 4 and 6.

Bacterial and fungal cultures were negative. Cryptococcal antigen was not detected in CSF. Serologic test results were negative for *Borrelia* spp., *Listeria* spp., parvovirus B19, measles virus, and galactomannan. PCR results for CSF were repeatedly negative for herpes simplex virus, varicella zoster virus, cytomegalovirus, *Toxoplasma gondii*, JC polyomavirus, human herpesvirus 6, Epstein-Barr virus, and mumps virus. PCR results and culture were negative for *Mycobacterium* spp. Serum samples were negative for antibodies against neuronal nuclear (Hu, Ri, and Yo) antigens. However, enterovirus RNA was detected by reverse transcription PCR in all CSF samples. Sequencing of the virion protein 1 gene obtained directly from RNA extracted from CSF identified the virus as coxsackievirus A16 (*3*).

Computed tomography scan of the brain on day 2 showed no abnormalities. However, brain magnetic resonance imaging scans on the third and fourth days showed bilateral, multiple, hyperintense white matter lesions in the periventricular region and cerebral hemispheres. Treatment was started empirically with broad-spectrum antimicrobial drugs and acyclovir; the acyclovir was stopped after infection with herpes simplex virus was excluded.

On day 4, imaging indicated development of aphasia and right hemiparesis without new lesions. The patient was transferred for mechanical ventilation after a grand mal seizure. Treatment with intravenous immune globulin (IVIG, 400 mg/kg) was started on day 4 and given for 5 consecutive days, which resulted in marked and continued neurologic improvement. Monthly doses of IVIG (500 mg/kg) resulted in normal serum IgG levels. Four months after initial examination, the virus has been cleared but the patient still has intermittent confusion and language defects. Treatment with IVIG will be continued for an additional 6 months.

Use of MAbs against CD20 B-cell antigen has become standard treatment for B-cell lymphomas and an increasing number of autoimmune disorders (*4*,*5*). However, resulting hypogammaglobulinemia predisposes patients to opportunistic infections, including progressive multifocal leukoencephalopathy and enterovirus infections (*2*,*6*). MAbs with enhanced activity against CD20 (e.g., obinutuzumab) have been developed. Obinutuzumab has been approved by the US Food and Drug Administration for treatment of chronic lymphocytic leukemia. Studies regarding the use of obinutuzumab for other B-cell malignancies are ongoing (*7*).

We anticipate that more cases of enteroviral encephalitis might develop, given the increasingly frequent use of MAbs against CD20 and widespread occurrence of enteroviruses. However, in view of the few reported cases (*2*), we also suspect that many cases remain undiagnosed despite availability of several pan-enterovirus diagnostic kits, which can detect low viral loads. Thus, clinicians should be suspicious of severe enterovirus infections in patients receiving MAbs.

Any patient receiving MAbs against CD20 who has neurologic symptoms should be screened for infection with enterovirus RNA. In contrast to JC polyomavirus–associated progressive multifocal leukoencephalopathy, enteroviral encephalitis can be successfully treated by early administration of IVIG, which might contain neutralizing antibodies, albeit in variable amounts (*8*). In the absence of double-blinded, placebo-controlled clinical studies of treatment for severe enterovirus infections, no specific antiviral therapy has been approved. However, 3 capsid inhibitors (pleconaril, pocapavir [V-073], and the pirodavir analog BTA-798) that show activity against enteroviruses are being developed (*9*). Pocapavir has potent activity against poliovirus and appears to be safe and well tolerated (*10*). In the United States, this drug is available by special request from the Food and Drug Administration.

Tom Eyckmans, Elke Wollants, Ann Janssens, Hélène Schoemans, Katrien Lagrou, Joost Wauters, and Johan Maertens

Author affiliation: University Hospitals Leuven, Leuven, Belgium

DOI: http://dx.doi.org/10.3201/eid2005.131766

References

1. Mao Q, Wang Y, Yao X, Bian L, Wu X, Xu M, et al. Coxsackievirus A16: epidemiology, diagnosis, and vaccine. Hum Vaccin Immunother. 2013;10. [Epub ahead of print].
2. Kassab S, Saghi T, Boyer A, Lafon ME, Gruson D, Lina B, et al. Fatal case of enterovirus 71 infection and rituximab therapy, France, 2012. Emerg Infect Dis. 2013;19:1345–7. http://dx.doi.org/10.3201/eid1908.130202
3. Thoelen I, Moës E, Lemey P, Mostmans S, Wollants E, Lindberg AM, et al. Analysis of the serotype and genotype correlation of VP1 and the 5′ noncoding region in an epidemiological survey of the human enterovirus B species. J Clin Microbiol. 2004;42:963–71. http://dx.doi.org/10.1128/JCM.42.3.963-971.2004
4. Griffin MM, Morley N. Rituximab in the treatment of non-Hodgkin's lymphoma: a critical evaluation of randomized controlled trials. Expert Opin Biol Ther. 2013;13:803–11. http://dx.doi.org/10.1517/14712598.2013.786698
5. Rosman Z, Shoenfeld Y, Zandman-Goddard G. Biologic therapy for

autoimmune diseases: an update. BMC Med. 2013;11:88 http://dx.doi.org/10.1186/1741-7015-11-88.
6. Casulo C, Maragulia J, Zelenetz AD. Incidence of hypogammaglobulinemia in patients receiving Rituximab and the use of intravenous immunoglobulin for recurrent infections. Clin Lymphoma Myeloma Leuk. 2013;13:106–11. http://dx.doi.org/10.1016/j.clml.2012.11.011
7. Goede V, Fischer K, Busch R, Engelke A, Eichhorst B, Wendtner CM, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. N Engl J Med. 2014. [Epub ahead of print]. http://dx.doi.org/10.1056/NEJMoa1313984
8. Wildenbeest JG, van den Broek PJ, Benschop KS, Koen G, Wierenga PC, Vossen AC, et al. Pleconaril revisited: clinical course of chronic enteroviral meningoencephalitis after treatment correlates with in vitro susceptibility. Antivir Ther. 2012;17:459–66. http://dx.doi.org/10.3851/IMP1936
9. Abzug MJ. The Enteroviruses: problems in need of treatments. J Infect. 2014;68:S108–14. http://dx.doi.org/10.1016/j.jinf.2013.09. 020
10. Hincks JR, Collet MS. Safety and pharmacokinetics of pocapavir, an oral antiviral candidate against poliovirus. In: Abstracts of the 53th Interscience Conference on Antimicrobial Agents and Chemotherapy, Denver, Colorado, Sep 10–13, 2013. Washington (DC): American Society for Microbiology; 2013. Abstract no. A-017d.

Address for correspondence: Johan Maertens, Department of Hematology, University Hospitals Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium; email: johan.maertens@uzleuven.be

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.

Serologic Evidence of Influenza A(H1N1) pdm09 Virus Infection in Northern Sea Otters

To the Editor: Sporadic epizootics of pneumonia among marine mammals have been associated with multiple animal-origin influenza A virus subtypes (*1*–*6*); seals are the only known nonhuman host for influenza B viruses (*7*). Recently, we reported serologic evidence of influenza A virus infection in free-ranging northern sea otters (*Enhydra lutris kenyoni*) captured off the coast of Washington, USA, in August 2011 (*8*). To investigate further which influenza A virus subtype infected these otters, we tested serum samples from these otters by ELISA for antibody-binding activity against 12 recombinant hemagglutinins (rHAs) from 7 influenza A hemagglutinin (HA) subtypes and 2 lineages of influenza B virus (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/5/13-1890-Techapp1.pdf). Estimated ages for the otters were 2–19 years (online Technical Appendix Table 2); we also tested archived serum samples from sea otters of similar ages collected from a study conducted during 2001–2002 along the Washington coast (*9*).

Of the 30 sea otter serum samples collected during 2011, a total of 21 (70%) had detectable IgG (≥200) for rHA of influenza A(H1N1)pdm09 virus (pH1N1) strain A/Texas/05/2009. Four of 7 serum samples that showed IgG ≥6,400 against pH1N1 rHA also showed low cross-reactivity (IgG 200) against rHA of A/Brisbane/59/2007, a previous seasonal influenza A(H1N1) virus (Figure, panel A; online Technical Appendix Table 1). No IgG was detected in any samples for any of the other 11 rHAs tested (IgG ≤100), and the sea otter serum samples collected during 2001–2002 did not react with any of the rHAs tested, including pH1N1 (IgG ≤100; Figure, panel A).

Next, we tested serum samples by using a hemagglutination inhibition (HI) assay with whole influenza virus to detect strain-specific antibodies that inhibit receptor binding. Of the 30 samples collected during 2011, a total of 22 (73%) showed HI antibody titers of ≥40 against pH1N1 virus. Titers against all other human and avian viruses tested were ≤10 for all samples by HI assay using turkey red blood cells (RBCs) (Figure, panel B; online Technical Appendix Table 3). No influenza A or B virus–specific HI antibodies were detected in the samples collected during 2001–2002 (data not shown). Although nasal swab specimens were collected from sea otters in the 2011 study, all specimens were negative for influenza virus by testing in embryonated eggs and by real-time PCR for detection of influenza A viral RNA (data not shown). These results suggest that sea otters were infected with influenza A virus sometime before the August 2011 sample collection date.

Although none of the 2011 samples showed HI titers to influenza A/duck/New York/96 (H1N1) virus (dk/NY/96) by testing using turkey RBCs (online Technical Appendix Table 2), titers against this strain were detected when using horse RBCs, which is a more sensitive means for the detection of mammalian antibodies against some avian influenza subtypes (*10*). Of the 22 samples that had HI titers ≥40 to pH1N1 virus, 16 also had HI titers ≥40 against dk/NY/96 by horse RBC HI assay (online Technical Appendix Table 2). However, titers against this strain were on average ≈4–8-fold lower than those for the pH1N1 virus strain, which suggests that that the titers against dk/NY/96 were the result of serologic cross-reactivity with avian- and swine-origin pH1N1 viruses.

To further test for cross-reactivity, 4 sea otter serum samples were

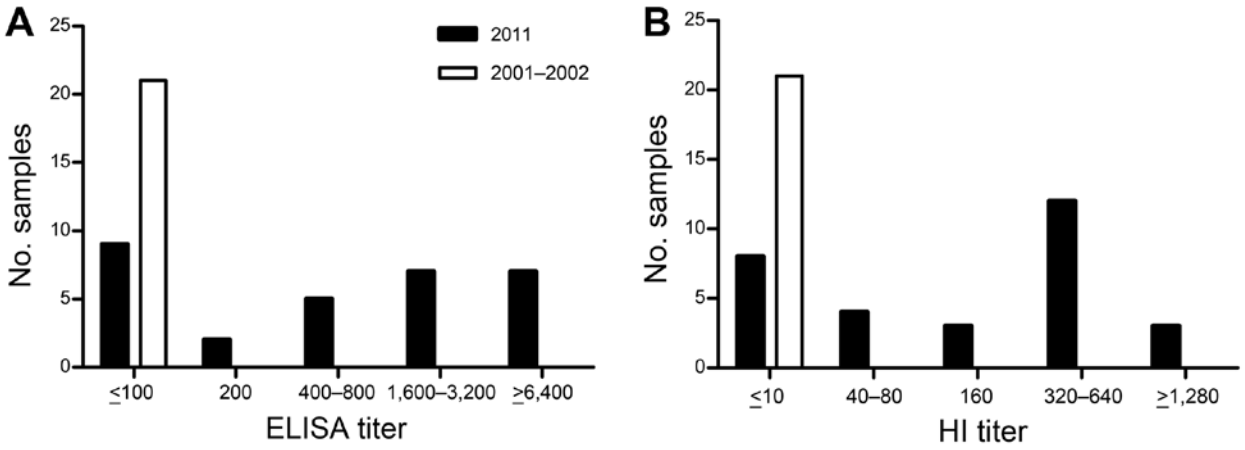


Figure. Results of ELISA and hemagglutination inhibition (HI) testing for influenza viruses in serum samples from northern sea otters captured off the coast of Washington, USA, during studies conducted in August 2011 (n = 30) and 2001–2002 (n = 21). A) IgG for influenza A(H1N1) pdm09 strain A/Texas/05/2009 detected by using standard indirect ELISA techniques with HRP-Protein A (Sigma, St. Louis, MO, USA). The ELISA titer was read as the reciprocal of the highest dilution of serum with an OD_{450nm} of ≥0.2 and 2-fold higher than the OD_{450nm} of control wells lacking serum. B) HI for influenza A(H1N1)pdm09 strain A/Mexico/4108/2009. HI titers were determined by using 0.5% turkey red blood cells (RBCs) for influenza A(H1N1)pdm09, seasonal influenza A(H1N1), influenza (H3N2), and influenza B viruses that circulated in North America during 2000–2011 and by using 1% horse RBCs supplemented with 0.5% BSA for avian influenza A(H1N1) virus strain A/duck/New York/96. HI assay was performed as described (www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en). OD, optical density.

adsorbed with purified pH1N1 and dk/NY/96 virions. Adsorption with pH1N1, but not dk/NY/96, removed HI antibodies to pH1N1, whereas adsorption with either virus removed HI antibodies against dk/NY/96 (online Technical Appendix Table 4). A comparison of amino acid sequences comprising the known HA antigenic sites on the pH1N1 structure confirmed high sequence identity and structural similarity with dk/NY/96 HA in Sa (12/13 aa residues) and Sb (8/12 aa residues) antigenic sites (data not shown). These results indicate that HI antibodies detected in sea otters are the result of pH1N1 virus infection but cross-react with the avian influenza A(H1N1) virus.

Although we cannot exclude the possibility that sea otters were infected with classical swine influenza A(H1N1) virus, which shares high HA genetic and antigenic similarity with pH1N1 virus, our serologic evidence is consistent with isolation of pH1N1 virus from northern elephant seals (*1*). Therefore, we conclude that these sea otters were infected with pH1N1 virus. The origin or transmission route of pH1N1 virus infection in sea otters remain unknown. Potential contact between northern elephant seals and sea otters is one possibility; elephant seals' summer feeding ranges and breeding areas along the Northeast Pacific coast overlap with areas where the Washington sea otter population is distributed (*1*).

In conclusion, our results show that sea otters are susceptible to infection with influenza A virus and highlight the complex nature of interspecies transmission of influenza viruses in the marine environment. Further surveillance, especially in other sea otter populations, is required to determine virus origin, potential pathogenesis, and consequences for the marine ecosystem.

Acknowledgments

We thank Heather Tatum, Leilani Thomas, and Peter Browning for specimen management and Tina Egstad, Katy Griffin, Renee Long, and Zac Najacht for technical assistance.

Sample collection was done in collaboration with United States Geological Survey–Alaska Science Center, Monterey Bay Aquarium, and Seattle Aquarium. Recombinant HAs with Histidine Tag from A/Japan/305/57 (H2N2), FR-700; A/Netherlands/219/2003 (H7N7), FR-71; A/Hong Kong/1073/99 (H9N2), FR-88; A/shorebird/DE/68/2004 (H13N9), FR-73; globular head domain HA1 rHAs of B/Brisbane/60/2008, FR-836; and B/Wisconsin/1/2010 FR-843, were obtained through the Influenza Reagent Resource, Influenza Division, World Health Organization Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention.

Zhu-Nan Li, Hon S. Ip, Jessica F. Trost, C. LeAnn White, Michael J. Murray, Paul J. Carney, Xiang-Jie Sun, James Stevens, Min Z. Levine, and Jacqueline M. Katz

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (Z.-N. Li, J.F. Trost, P.J. Carney, X.-J. Sun, J. Stevens, M.Z. Levine, J.M. Katz); United States Geological Survey National Wildlife Health Center, Madison, Wisconsin, USA (H.S. Ip, C.L. White); and Monterey Bay Aquarium, Monterey, California, USA (M.J. Murray)

DOI: http://dx.doi.org/10.3201/eid2005.131890

References

1. Goldstein T, Mena I, Anthony SJ, Medina R, Robinson PW, Greig DJ, et al. Pandemic H1N1 influenza isolated from free-ranging Northern Elephant Seals in 2010 off the central California coast. PLoS ONE. 2013;8:e62259. http://dx.doi.org/10.1371/journal.pone.0062259
2. Hinshaw VS, Bean WJ, Geraci J, Fiorelli P, Early G, Webster RG. Characterization of two influenza A viruses from a pilot whale. J Virol. 1986;58:655–6.
3. Hinshaw VS, Bean WJ, Webster RG, Rehg JE, Fiorelli P, Early G, et al. Are seals frequently infected with avian influenza viruses? J Virol. 1984;51:863–5.
4. Geraci JR, St Aubin DJ, Barker IK, Webster RG, Hinshaw VS, Bean WJ, et al. Mass mortality of harbor seals: pneumonia associated with influenza A virus. Science. 1982;215:1129–31. http://dx.doi.org/10.1126/science.7063847
5. Anthony SJ, St Leger JA, Pugliares K, Ip HS, Chan JM, Carpenter ZW, et al. Emergence of fatal avian influenza in New England harbor seals. MBio. 2012;3:e00166–12. http://dx.doi.org/10.1128/mBio.00166-12
6. Callan RJ, Early G, Kida H, Hinshaw VS. The appearance of H3 influenza viruses in seals. J Gen Virol. 1995;76:199–203. http://dx.doi.org/10.1099/0022-1317-76-1-199
7. Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA. Influenza B virus in seals. Science. 2000;288:1051–3. http://dx.doi.org/10.1126/science.288.5468.1051
8. White CL, Schuler KL, Thomas NJ, Webb JL, Saliki JT, Ip HS, et al. Pathogen exposure and blood chemistry in the Washington, USA population of northern sea otters (*Enhydra lutris kenyoni*). J Wildl Dis. 2013;49:887–99. http://dx.doi.org/10.7589/2013-03-053
9. Brancato MS, Milonas L, Bowlby CE, Jameson R, Davis JW. Chemical contaminants, pathogen exposure and general health status of live and beach-cast Washington sea otters (*Enhydra lutris kenyoni*). Marine Sanctuaries Conservation Series ONMS-09–01. Silver Spring (MD): US Department of Commerce, National Oceanic and Atmospheric Administration, Office of National Marine Sanctuaries; 2009. p. 181.
10. Stephenson I, Wood JM, Nicholson KG, Zambon MC. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J Med Virol. 2003;70:391–8. http://dx.doi.org/10.1002/jmv.10408

Address for correspondence: Jacqueline M. Katz, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G16, Atlanta, GA 30333, USA: email: jmk9@cdc.gov

New Variant of Porcine Epidemic Diarrhea Virus, United States, 2014

To the Editor: Porcine epidemic diarrhea (PED) was first reported in the United Kingdom in 1971 (*1*). The disease was characterized by severe enteritis, vomiting, watery diarrhea, dehydration, and a high mortality rate among swine. Subsequently, the causative agent of PED was identified as porcine epidemic diarrhea virus (PEDV), which belongs to the family *Coronaviridae* (*2*) and contains an enveloped, single-stranded positive-sense RNA genome. PEDV has been reported in many other countries, including Germany, France, Switzerland, Hungary, Italy, China, South Korea, Thailand, and Vietnam (*3*) and was first identified in the United States in May 2013. By the end of January of 2014, the outbreak had occurred in 23 US states, where 2,692 confirmed cases (www.aasv.org/news/story.php?id = 6989) caused severe economic losses. Recent studies have shown that all PEDV strains in the United States are clustered together in 1 clade within the subgenogroup 2a and are closely related to a strain from China, AH2012 (*4*,*5*).

In the state of Ohio, the first PED case was identified in June of 2013; since then, hundreds of cases have been confirmed by the Animal Disease Diagnostic Laboratory of the Ohio Department of Agriculture. In January of 2014, samples from pigs with unique disease, suspected to be PED, were submitted to this laboratory. Sows were known to be infected, but piglets showed minimal to no clinical signs and no piglets had died.

According to real-time reverse transcription PCR, all samples from the piglets were positive for PEDV. Subsequently, the full-length genome sequence of PEDV (OH851) was determined by using 19 pairs of oligonucleotide primers designed from alignments of the available genomes from PEDVs in the United States (*6*,*7*). On the basis of BLAST (http:blast.ncbi.nlm.nih.gov/Blast.cgi) searches, strain OH851 showed 99% and 97% nt identity to PEDVs currently circulating in the United States (Colorado, Iowa, Indiana, Minnesota) for the whole genome and the full-length spike (S) gene, respectively. By distinct contrast, strain OH851 showed only 89% or even lower nucleotide identity to PEDVs currently circulating in the United States in the first 1,170 nt of the S1 region. In that region, nucleotide similarity to that of a PEDV strain from China (CH/HBQX/10, JS120103) was 99%, suggesting that strain OH851 is a new PEDV variant. Phylogenetic analysis of the complete genome indicated that the novel OH851 PEDV is clustered with other strains of PEDV currently circulating in United States, including another stain from Ohio, OH1414 (Figure, panel A). However, phylogenetic analysis of the full-length S gene showed that strain OH851 is clustered with other strains of PEDV from China and most closely related to a PEDV strain from China, CH/HBQX/10 (*8*), but distantly related to other PEDV strains currently circulating in the United States and strain AH2012 (Figure, panel B). This finding strongly suggests that strain OH851 is a variant PEDV. In comparison with the S gene of other strains from the United States, the S gene of strain OH851 has 3 deletions (a 1-nt deletion at position 167, a 11-nt deletion at position 176, and a 3-nt deletion at position 416), a 6-nt insertion between positions 474 and 475,

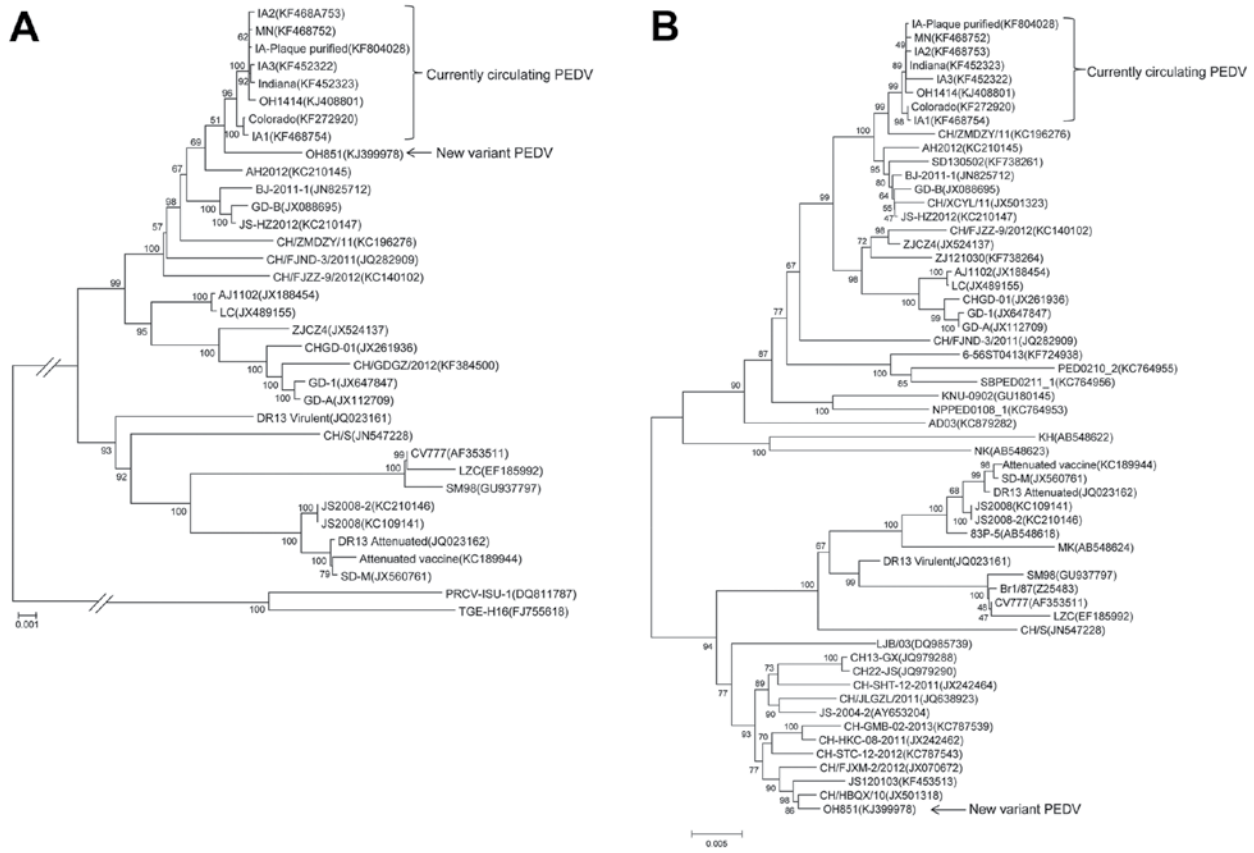


Figure. Phylogenetic tree of the whole-genome sequences of 33 strains of porcine epidemic diarrhea virus (PEDV) (A) and of spike protein nucleotide sequences of 56 strains of PEDV (B), including the new variant PEDV (OH851) and 8 PEDV strains currently circulating in the United States. The dendrogram was constructed by using the neighbor-joining method in MEGA version 6.05 (www.megasoftware.net). Bootstrap resampling (1,000 replications) was performed, and bootstrap values are indicated for each node. Reference sequences obtained from GenBank are indicated by strain name and accession number. Scale bars indicate nucleotide substitutions per site.

and several mutations mainly located in the first 1,170 nt of the S1 region.

It is highly possible that the sequence deletions, insertion, and mutations found in variant strain OH851 might have contributed to the reduced severity of the clinical disease in the piglets. More animal studies are needed to test this hypothesis. The unique deletion and insertion feature also represents a target for diagnostic assays to differentiate between currently circulating PEDV strains and new variants.

The low nucleotide identity in the 5′-end S1 region (first 1,170 nt) region and high nucleotide identity in the non-5′-end S1 region of the variant strain, compared with that of the PEDVs currently circulating in the United States, suggest that this new PEDV variant might have evolved from a recombinant event involving a strain from China. Because the new variant does not cause severe clinical disease, including death, the novel virus is a potential vaccine candidate that could protect the US swine industry from the infection caused by the virulent strain of PEDV currently circulating in United States.

Leyi Wang,[1] Beverly Byrum, and Yan Zhang[1]

Author affiliation: Ohio Department of Agriculture Reynoldsburg, Ohio, USA

DOI: http://dx.doi.org/10.3201/eid2005.140195

[1]These authors were co–principal investigators.

References

1. Wood EN. An apparently new syndrome of porcine epidemic diarrhoea. Vet Rec. 1977;100:243–4. http://dx.doi.org/10.1136/vr.100.12.243
2. Pensaert MB, de Bouck P. A new coronavirus-like particle associated with diarrhea in swine. Arch Virol. 1978;58:243–7. http://dx.doi.org/10.1007/BF01317606
3. Song D, Park B. Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. Virus Genes. 2012;44:167–75. http://dx.doi.org/10.1007/s11262-012-0713-1
4. Huang YW, Dickerman AW, Pineyro P, Li L, Fang L, Kiehne R, et al. Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. mBio 2013;4:e00737–13. http://dx.doi.org/10.1128/mBio.00737-13
5. Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D, et al.

Emergence of porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J Vet Diagn Invest. 2013;25:649–54. http://dx.doi.org/10.1177/1040638713501675

6. Hoang H, Killian ML, Madson DM, Arruda PH, Sun D, Schwartz KJ, et al. Full-length genome sequence of a plaque-cloned virulent porcine epidemic diarrhea virus isolate (USA/Iowa/18984/2013) from a midwestern U.S. swine herd. Genome Announcements. 2013;1: pii: e01049-13. doi:10.1128/genomeA.01049-13
7. Marthaler D, Jiang Y, Otterson T, Goyal S, Rossow K, Collins J. Complete genome sequence of porcine epidemic diarrhea virus strain USA/Colorado/2013 from the United States. Genome Announcements. 2013; 1. pii: e00555-13. http://dx.doi.org/10.1128/genomeA.00555-13
8. Zheng FM, Huo JY, Zhao J, Chang HT, Wang XM, Chen L, et al. Molecular characterization and phylogenetic analysis of porcine epidemic diarrhea virus field strains in central China during 2010–2012 outbreaks [in Chinese]. Bing Du Xue Bao. 2013; 29:197–205.

Address for correspondence: Yan Zhang, Animal Disease Diagnostic Laboratory, Ohio Department of Agriculture, 8995 East Main St, Building 6, Reynoldsburg, OH 43068, USA: email: yzhang@agri.ohio.gov

B.J. Adams. *With Apologies to Arcimboldo* (detail), 2009. Flower petals on radiation mask, 14″ x 13″ x 12″. http://courageunmasked.org/

Courage Unmasked

Sharon Bloom

Lying supine, the technician uses my mask to bolt me to the treatment table.
After he leaves the room, I feel alone, and at first, afraid.
I see and hear the radiation machine move around me, but feel nothing.
Minutes later, the door opens, and the mask is unbolted.
This process is repeated almost daily for 2 months…
After 2 weeks, the skin on my neck and inside my mouth becomes red, sore, and dry.
My sense of taste changes, it becomes difficult to eat.
By the end, the technician has become my trusted friend,
I can not predict the future, but I feel less afraid, my
Courage Unmasked.

—Cookie Kerxton (pers. comm., February 2014)

Radiation treatment for head and neck cancer involves the use of custom-molded plastic mesh masks placed over the head and shoulders to immobilize the patient and facilitate the stereotactic positioning of radiation beams. This month's cover art was selected from the "Courage Unmasked" project, which was founded by Cookie Kerxton, a head and neck cancer survivor and artist, who during one such treatment session envisioned the use of art to both raise awareness of and support fellow patients in need. After the discarded masks have been transformed into works of art, they are exhibited and auctioned off.

Fiber artist B.J. Adams was one of more than 100 artists invited to create art from used radiation masks with materials of their choice. Inspired by Italian artist Giuseppe Arcimboldo (1527–1593), who created portraits by using objects, such as flowers, vegetables, and fruit, Adams chose brightly colored artificial flowers and insects to create a spring-like mask to symbolize rebirth and rejuvenation. Adams was born in California and studied fine art; she has been working in mixed media for ≈60 years and lives in Washington, DC.

In some countries, human papillomavirus (HPV) is found in most oropharyngeal cancers, a subset of head and neck cancers. New data from the United States show that the association of HPV with oropharyngeal cancers is more common than previously believed; furthermore, the most common HPV types detected in these cancers are types prevented by available vaccines. It is thus conceivable that HPV vaccines developed to prevent cervical cancer might also prevent some oropharyngeal cancers.

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: http://dx.doi.org/10.3201/eid2005.AC2005

Acknowledgments

I thank Marian McDonald for feedback on a draft. Proceeds from the sale of masks support the nonprofit Help for Head and Neck Cancer Fund, which grants small subsidies to persons who have head and neck cancer and who are in financial need (more information at 9114hnc.org). Additional artwork by B.J. Adams can be found at wwwbjadamsart.com. Readers interested in seeing how masks are created and used for patient care can view the patient instructional video produced by the Radiation Medicine Program, Princess Margaret Cancer Centre, University Health Network (www.youtube.com/watch?v=-dWm40P6elM).

Bibliography

1. Steinau M, Saraiya M, Goodman MT, Peters ES, Watson M, Cleveland JL, et al. Oropharyngeal cancer cells before vaccine introduction, United States. Emerg Infect Dis. 2014;20:822–8.
2. Potter P. The extraordinary nature of illusion. Emerg Infect Dis. 2008;14:1829–30. http://dx.doi.org/10.3201/eid1411.AC1411
3. Morley L, McAndrew A, Tse K, Rakaric P, Cummings B, Cashell A. Patient and staff assessment of an audiovisual education tool for head and neck radiation therapy. J Cancer Educ. 2013;28:474–80. http://dx.doi.org/10.1007/s13187-013-0489-6

Address for correspondence: Sharon Bloom, Center for Global Health, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E41, Atlanta, GA 30333, USA; email: sbloom@cdc.gov

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to **www.medscape.org/journal/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.org. If you are not registered on Medscape.org, 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 may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Outbreaks of *Kingella kingae* Infections in Daycare Facilities

CME Questions

1. You are seeing a 3-year-old boy who was just diagnosed with osteomyelitis of the left tibia. The child attends day care 5 times per week. You consider whether this patient might have a hematogenous infection with *Kingella kingae*. Besides the bones, this organism displays a tropism for what other anatomic site?

A. Esophagus
B. Kidneys
C. Brain
D. Heart

2. What should you consider regarding the clinical presentation of invasive infection with *K. kingae*?

A. Most children with invasive infection have multiple chronic medical conditions
B. Nearly all children have fever
C. A normal serum C-reactive protein level rules out the possibility of *K. kingae* osteomyelitis
D. The typical presentation of *K. kingae* bacteremia makes the diagnosis easy to miss

3. You initiate laboratory work to identify possible *K. kingae* infection for this patient. Which of the following statements regarding the diagnostic process for *K. kingae* infections is most accurate?

A. Less than 1% of children between 12 and 24 months old harbor the *K. kingae* bacterium
B. Bone exudates in particular grow well on routine solid culture media
C. Medium consisting of blood-agar with added 2 mg/mL of vancomycin should be avoided in respiratory cultures
D. Nucleic acid amplification assays have improved the sensitivity of detection of *K. kingae* and reduce the time needed for diagnosis

4. The patient is diagnosed with *K. kingae* osteomyelitis. What should you consider regarding a possible outbreak of infection in his day care class?

A. Previous data suggest that the risk for another *K. kingae* infection within 1 month exceeds 10%
B. Isoniazid is the drug of choice as prophylaxis
C. Azithromycin is the drug of choice as prophylaxis
D. Multiple antibiotic prescriptions are associated with 100% eradication of *K. kingae*

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

Upcoming Issue

Effects of Q Fever during Pregnancy on Mother and Child

Short-Term Malaria Reduction by Single-Dose Azithromycin during Mass Drug Administration for Trachoma, Tanzania

Human Polyomavirus 9 Infection in Kidney Transplant Patients

Oral Fluid Testing for Pertussis, England and Wales

Importation of *Falciparum malaria* to Guatemala from Democratic Republic of the Congo

Clinical and Laboratory Characteristics of Patients with Mild to Moderate Primary Pulmonary Coccidioidomycosis

Dengue Virus Type-3, South Pacific Islands, 2013

Identification of Possible Virulence Marker from *Campylobacter jejuni* Isolates

MERS Coronaviruses in Dromedary Camels, Egypt

Timeliness of Yellow Fever Surveillance, Central African Republic

Sequential Infection with 2 Norovirus Genotypes

New Hepatitis E Virus Genotype in Camel, the Middle East

Co-infection with *Mycobacterium ulcerans* and *Mansonella perstans* among Buruli Ulcer Patients, Ghana

Human Infection with MERS Coronavirus after Exposure to Infected Camels, Saudi Arabia, 2013

Unraveling the Mysteries of Middle East Respiratory Syndrome Coronavirus

Genetic Relatedness of Dolphin Rhabdovirus and Fish Rhabdoviruses

Schmallenberg Virus Circulation in High Mountain Ecosystem, Northeastern Spain

Bartonella spp. and *Yersinia pestis* Reservoirs, Cusco, Peru

Videodermatoscopic Diagnosis of Trombiculosis

Novel Henipa-like Virus, Mojiang Paramyxovirus, in Rats, China, 2012

Streptococcus suis Infection and Malignancy in Man, Spain

Human Granulocytic Anaplasmosis Acquired in Scotland, 2013

Novel Reassortant Influenza A(H5N8) Viruses, South Korea, 2014

Complete list of articles in the June issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

May 10–13, 2014
ECCMID
European Congress of Clinical Microbiology and Infectious Diseases
Barcelona, Spain
http://www.eccmid.org/

May 17–20, 2014
114th General Meeting
American Society for Microbiology
Boston, MA
http://www.asm.org/asm2014/

June 24–27, 2014
EMBO conference on Microbiology After the Genomics Revolution – Genomes 2014
Institut Pasteur, Paris
http://www.genomes-2014.org

July 27–August 1, 2014
IUMS 2014
International Union of Microbiological Societies
Montréal, Canada
http://www.montrealiums2014.org/side_registration/secure_online_registration_e.shtml

September 5–9, 2014
ICAAC 2014
Interscience Conference on Antimicrobial Agents and Chemotherapy
Washington, DC
http://www.icaac.org

October 8–12, 2014
ID Week 2014
Philadelphia, PA
http://www.idweek.org/

October 31–November 3, 2014
IMED 2014
Vienna, Austria
http://imed.isid.org

November 15–19, 2014
APHA 142nd Annual Meeting & Expo
New Orleans, LA
http://www.apha.org/meetings/

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

CDC PROVIDES INFORMATION ABOUT MRSA SKIN INFECTIONS.

Visit www.cdc.gov/MRSA or call 1-800-CDC-INFO (800-232-4636)
TTY: (888) 232-6348 to order provider materials including:

- Clinician guidelines
- Evaluation & treatment recommendations
- Patient education materials
- Posters
- Fact sheets
- Flyers

cdcinfo@cdc.gov

Developed with support from the CDC Foundation through an educational grant from Pfizer Inc.

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

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. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

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. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may 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.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 biographical sketch. Dispatches are updates on infectious disease trends and research that 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.

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. Include biographical sketch.

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. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.