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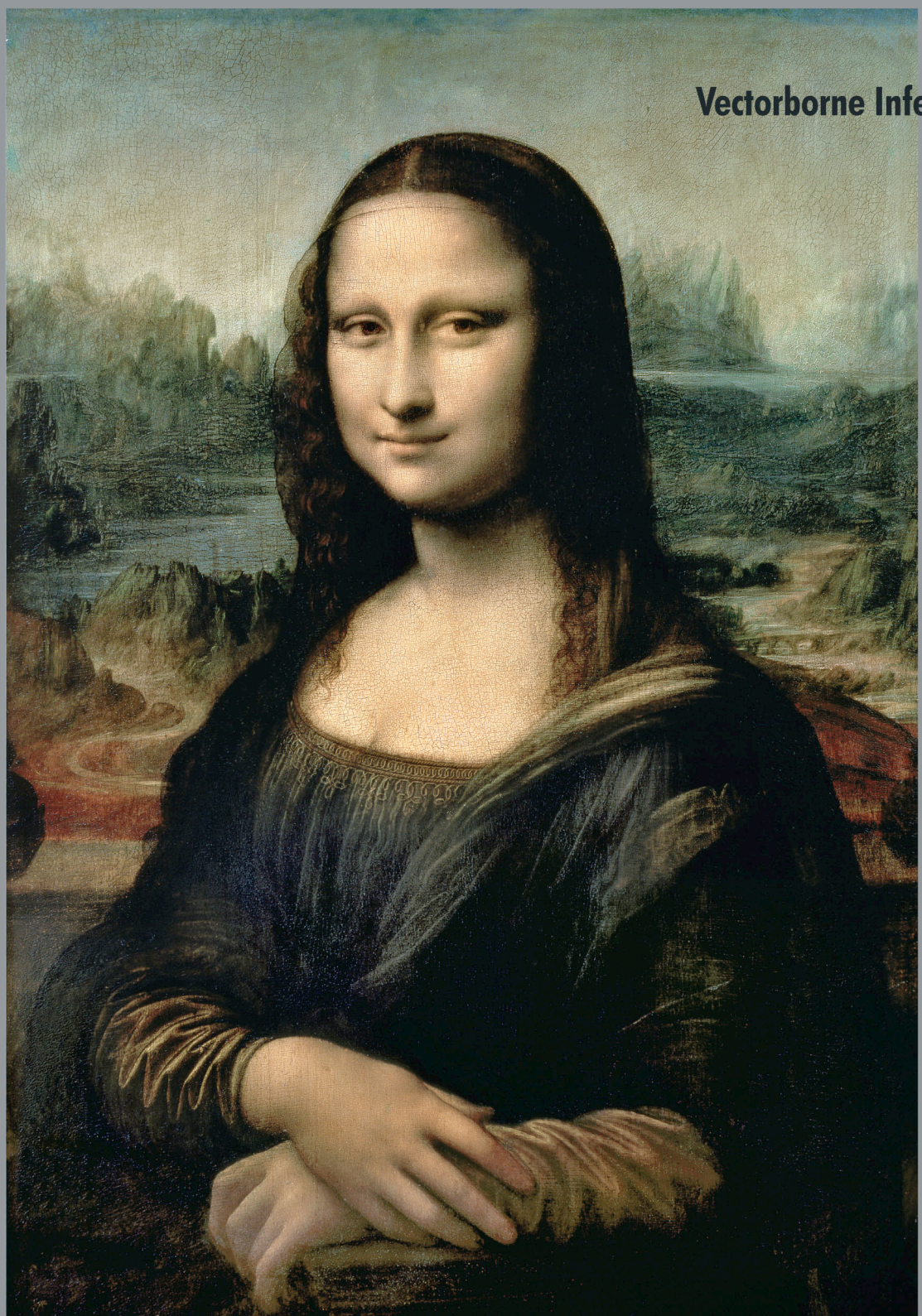

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Mona Lisa (c.1503–1506).
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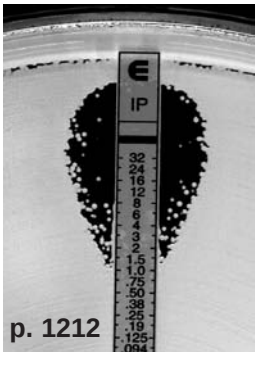

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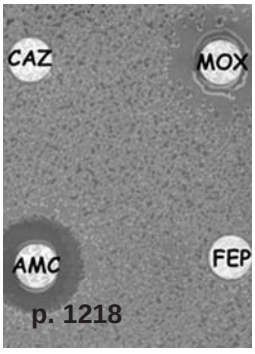

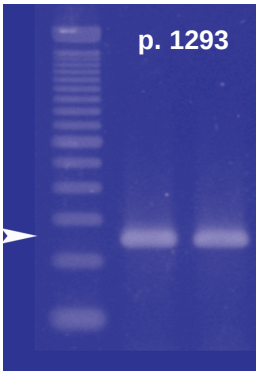

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Invasive *Enterobacter sakazakii* Disease in Infants

Anna B. Bowen* and Christopher R. Braden*

Enterobacter sakazakii kills 40%–80% of infected infants and has been associated with powdered formula. We analyzed 46 cases of invasive infant *E. sakazakii* infection to define risk factors and guide prevention and treatment. Twelve infants had bacteremia, 33 had meningitis, and 1 had a urinary tract infection. Compared with infants with isolated bacteremia, infants with meningitis had greater birthweight (2,454 g vs. 850 g, p = 0.002) and gestational age (37 weeks vs. 27.8 weeks, p = 0.02), and infection developed at a younger age (6 days vs. 35 days, p<0.001). Among meningitis patients, 11 (33%) had seizures, 7 (21%) had brain abscess, and 14 (42%) died. Twenty-four (92%) of 26 infants with feeding patterns specified were fed powdered formula. Formula samples associated with 15 (68%) of 22 cases yielded *E. sakazakii*; in 13 cases, clinical and formula strains were indistinguishable. Further clarification of clinical risk factors and improved powdered formula safety is needed.

Enterobacter sakazakii, a gram-negative bacillus, is a rare cause of bloodstream and central nervous system infections (*1–8*). The organism has also been associated with necrotizing enterocolitis; however, it has not been firmly established as a causative agent (*8–10*). Reported outcomes are often severe: seizures; brain abscess; hydrocephalus; developmental delay; and death in as many as 40%–80% of cases (*11*). Premature infants are thought to be at greater risk than more mature infants, other children, or adults, and outbreaks of disease have occurred in hospital units for newborns (*1,2,4,6,8–10,12*). Infant infections with *E. sakazakii* have been associated with contaminated powdered formula products, but other environmental sources of contamination are possible (*1,3,9,10,13*). In this analysis, we attempt to more clearly define the host risk factors and disease course to refine prevention and treatment efforts.

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Methods

We reviewed the literature for reports of *E. sakazakii* disease in infants. Using medical subject heading terms "*E. sakazakii*" or "*Enterobacter*" in combination with "newborn," "infant," or "meningitis," we searched PubMed and examined the bibliographies of resulting articles. Finally, we reviewed *E. sakazakii* case consultations conducted by the Centers for Disease Control and Prevention (CDC) from 1998 to 2005 and reviewed results of a French outbreak reported to CDC in 2005 (*14*).

We defined a case as an infant (<12 months of age) with *E. sakazakii* cultured from an area of the body that is normally sterile: tissue, blood, cerebrospinal fluid, or urine aspirated through the bladder wall. Infants with cultures of cerebrospinal fluid or brain abscesses yielding *E. sakazakii* were considered to have meningitis. Because bacteremia is usually an intermediate step during the process of developing meningitis, patients with both bacteremia and meningitis were included in the meningitis group. Bacteremia was defined as *E. sakazakii* grown from the blood of a case-patient without evidence of meningitis.

Infants with gestational ages <37 weeks, or those reported to be premature, were considered premature. A gestational age of 40 weeks was assigned to 3 (27%) of 11 infants with bacteremia and 4 (14%) of 28 infants with meningitis who were reported as born following term gestation without a specific gestational age mentioned. We defined birthweight <2,500 g as low birthweight (LBW), <1,500 g as very low birthweight (VLBW), and <1,000 g as extremely low birthweight (ELBW).

Descriptive analysis was performed by using SAS software (SAS Institute, Cary, NC, USA). Groups were compared by using 2-tailed Fisher exact test.

Results

Forty-six infants met the case definition (Table 1). Reported onset years ranged from 1958 to 2005; 32 (70%) cases were reported during the second half of this period.

Table 1. Cases of invasive *Enterobacter sakazakii* disease among infants

Year of report	Location	No. cases	Reference
1961	England	2	(*15*)
1965	Denmark	1	(*5*)
1979	Georgia, USA	1	(*7*)
1981	Indiana, USA	1	(*16*)
	Oklahoma, USA	1	
1983	Netherlands	8	(*8*)
1985	Missouri, USA	1	(*17*)
1988	USA	2	
1989	Portugal	1	(*18*)
	Iceland	3	(*3*)
	Tennessee, USA	3	(*10*)
1990	Maryland, USA	1	(*19*)
1991	Ohio, USA	1	(*20*)
2000	North Carolina, USA	1	
2001	Israel	2	(*2,4*)
	Belgium	1	(*9*)
2002	Israel	2	(*4*)
	Tennessee, USA	1	(*1*)
	Wisconsin, USA	1	CDC, unpub. data
2003	USA	6	CDC, unpub. data
2004	France	2	(*14*)
	USA	2	CDC, unpub. data
2005	USA	2	CDC, unpub. data

Cases were reported in 7 countries in North America, Europe, and the Middle East. Thirty-three (72%) infants had meningitis, 12 (26%) had bacteremia, and 1 (2%) had a urinary tract infection.

Clinical characteristics were available for a subset of cases (Table 2). Eight (40%) of 20 infants for whom data were available were delivered by cesarean section. Twenty-nine (69%) of 42 infants experienced disease onset within a hospital. Gestational duration was available for 38 infants; 21 (55%) were born prematurely, and the median gestational age overall was 36 weeks (range 23.5–40 weeks). The median birthweight was 2,063 g (range 540–3,401); 18 (56%) of 32 infants had LBW; 9 (28%) of these met the definition for VLBW, and 7 (22%) met the definition for ELBW. Median age at the time of infection onset was 8.5 days (range 2–300).

Although the proportion of infants who experienced nosocomial disease onset was not significantly different between the meningitis and bacteremia groups, other infant characteristics differed by site of infection (Figure). The median gestational ages of infants with meningitis and bacteremia were 37 and 27.8 weeks, respectively ($p = 0.02$). Median birthweights were 2,454 g and 850 g, respectively ($p = 0.002$). However, median age at infection onset was 6 days in the group with meningitis and 35 days in the group with bacteremia ($p < 0.0001$). Thirty (94%) of 32 infants with meningitis, but only 2 (18%) of 11 infants with bacteremia, were <28 days old when infection was detected. One infant (8%) with bacteremia died; this infant also had necrotizing enterocolitis. The single infant with a urinary tract infection recovered without complication. In contrast, 14 (42%) of 33 infants with meningitis died. Of 19 surviving infants, only those with meningitis suffered adverse outcomes, including brain abscess (21%, $p = 0.2$), developmental delays (53%, $p = 0.004$), motor impairment (21%, $p = 0.3$), and ventricular shunt placement (42%, $p = 0.01$); 74% experienced at least one of these outcomes.

Feeding practices were described for 26 infants. Twenty-four (92%) received a powdered formula product, including an infant who received powdered breast milk fortifier but no powdered infant formula; 1 additional infant received formula of an unspecified type. *E. sakazakii* was cultured from formula associated with 15 (68%) of 22 cases investigated. Isolates were obtained from prepared formula, opened formula tins, and previously unopened formula tins associated with 2, 6, and 7 cases, respectively. Thirteen (87%) of the 15 formula isolates were indistinguishable from the corresponding clinical strain by biotype or genotype; multiple formula manufacturers were implicated. In one of the remaining cases, multiple *E. sakazakii* strains were recovered from powdered formula, but none matched the clinical isolate by pulsed-field gel electrophoresis (CDC, unpub. data). In the other case, 2 *E. sakazakii* strains were isolated from blood and from rectal swabs; a third strain, as determined by arbitrarily primed PCR, was recovered from the powdered infant formula (*9*).

Discussion

Although numerous reports include infants with *E. sakazakii* isolated from nonsterile sites, such as respiratory secretions or stool, 46 infants identified from the literature and CDC sources met the case definition for this analysis (*1,9,10,12*). Of the infants with sterile-site infection, 72% had meningitis. Contrary to previous characterizations of *E. sakazakii* disease, we found that infants with meningitis and bacteremia alone fell into 2 distinct groups. Those in whom meningitis developed tended to be of greater gestational age and birthweight than those with bacteremia alone. In fact, infants in whom meningitis developed tended to attain near-term gestational age and birthweight. In contrast, infants in whom bacteremia alone developed tended to be born very prematurely and have ELBW. A second major difference between the group with meningitis and the group with bacteremia was the infants' chronological ages. Infants with meningitis were generally <1 week of age at the onset of infection with *E. sakazakii*, whereas infants with bacteremia had generally surpassed the neonatal period at the onset of their disease. Rates of adverse outcome also differed between the 2 groups, although this was not unexpected. Most infants with

Table 2. Characteristics of infants in the *Enterobacter sakazakii* case series

Characteristic	Published cases, n/N	Unpublished cases, n/N	Overall %
Male	15/30	6/9	54
Cesarean delivery	6/13	2/7	40
Nosocomial onset	24/31	5/11	69
Premature birth	17/29	4/9	55
Birthweight*			
LBW	16/27	2/5	56
VLBW	8/27	1/5	28
ELBW	6/27	1/5	22

*LBW, low birth weight; VLBW, very low birth weight; ELBW, extremely low birth weight.

E. sakazakii bacteremia fared better than those with meningitis. Among those in whom meningitis developed, rates of adverse outcome were similar to those reported in the literature: In this case series, 74% of meningitis survivors experienced an adverse neurologic outcome, while other studies cite adverse outcome in 20%–78% of neonatal or infant meningitis survivors (*21–23*).

The division of the infant population into 2 distinct groups occurred for unclear reasons. That infants <1 week of age comprised the meningitis group was not surprising; the disparities in other infant characteristics, however, are not intuitive. Since infants in the 2 groups experienced similar rates of nosocomial disease onset, the infants with bacteremia were unlikely to have simply received treatment earlier in the disease course than the infants with meningitis. Other risk factors are probable.

One likely set of risk factors is infant formula-feeding practices. Powdered infant formula is a demonstrated source of *E. sakazakii* infection. A microbiologic survey of powdered infant formulas published in 1988 found *E. sakazakii* in 20 (14%) of 141 samples tested (*24*). However, a survey of 82 powdered infant formula samples in 2003 yielded *E. sakazakii* in 2 (2.4%), which suggests that recent rates of powdered formula contamination may be lower (*25*). Powdered formula has also been implicated both epidemiologically and microbiologically as a vehicle in several cases of *E. sakazakii* disease in infants (*1,3,9,10,13*). Although we could not explore feeding exposures fully with the data available in this series, infant feeding practices may relate to the differences we describe in chronological age of infants at onset of meningitis and bacteremia. Infants with nearly normal gestational ages and birthweights are likely to be tended in normal newborn nurseries during the first 24–72 hours of life. In such nurseries, powdered formula is frequently given to babies who are not breast-fed, and it may also be used as a supplement by mothers who have chosen to breast-feed. Since infants are at highest risk for meningitis during the first several weeks of life, possibly because of immaturity of the blood-brain barrier, exposures to *E. sakazakii* in powdered formula or other sources during this time may quickly lead to central nervous system disease (*26,27*).

Conversely, in the intensive care settings where immature and low birthweight infants are tended, babies are not often fed powdered formula in the first few weeks of life. They may be given parenteral nutrition initially and may be fed breast milk from their own mothers or from a banked source when they do begin enteral feeds. If breast milk is not available, they are more likely to be given sterile, premixed infant formula than powdered formula, since standard preterm infant formula is only available in this form (*28*). Powdered breast milk fortifiers are not introduced until premature infants tolerate full-volume feeds,

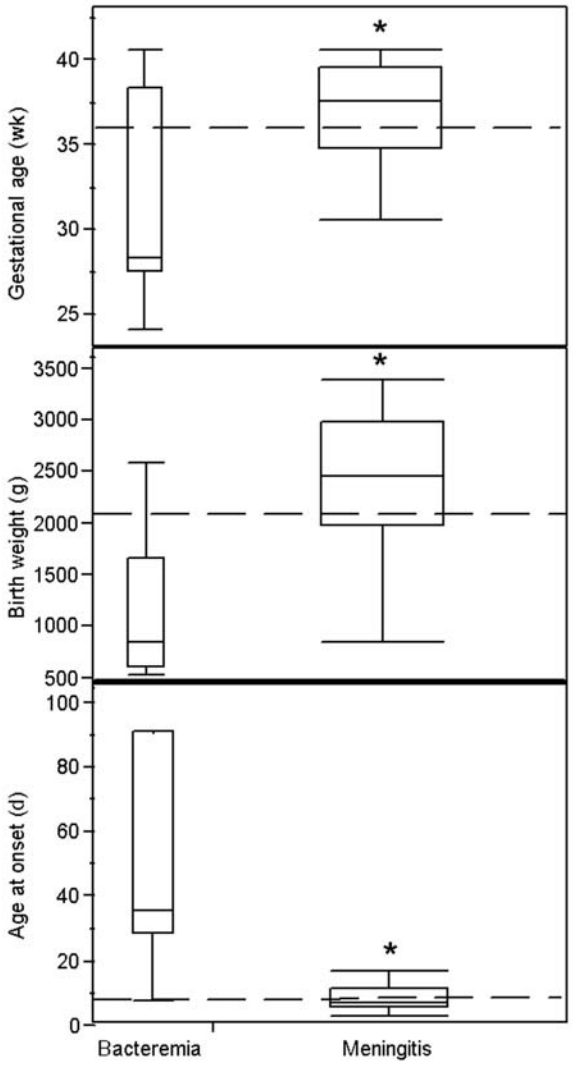

Figure. Box plot with each box indicating range (vertical lines), first and third quartiles (lower and upper boundaries of box, respectively), and median value (horizontal solid line) for gestational age in weeks, birth weight in grams, and age of onset in days for infants with bacteremia only or meningitis. The dotted lines indicate median values for all cases. *Significantly different values (α=0.05) between groups.

which may not occur for days or weeks after birth. Thus, infants in intensive care settings may not be exposed to nonsterile formula until they are more mature, which would lead to a greater proportion of *E. sakazakii* bacteremia than meningitis in this group if powdered formula is a source of infection. In this series, we were unable to explore the roles of indwelling enteric tubes, prior gastrointestinal surgery, and antecedent antacid or antimicrobial drug use as risk factors for infection.

While the reservoir for *E. sakazakii* is unknown, several environmental sources have been reported. *E. sakazakii* has been isolated from factories used to produce milk powder, chocolate, cereal, potato flour, spices, and pasta (*29*). It also has been isolated from household vacuum cleaner bags and from the guts of the stable fly, *Stomoxys calcitrans*, and the Mexican fruit fly, *Anastrpha ludens* (*29–31*). The relationship between these potential environmental sources and infant disease remains unclear. Although a human vaginal tract culture yielding *E. sakazakii* has been reported, vertical transmission is unlikely because nearly half of infants with *E. sakazakii* disease in this review were delivered by cesarean section, and symptoms developed in only 1 infant earlier than 3 days of age (*32*).

Our analyses were constrained by the use of retrospective and often incomplete data. Although cases with more severe outcomes might have been investigated and published more frequently than uncomplicated cases, this possible bias would not likely affect the representativeness of baseline infant characteristics. Assigning a gestational age of 40 weeks to term infants without a reported gestational age may have falsely elevated the median gestational ages we report, since most term infants are born at <40 weeks' gestation (*33*). However, a greater proportion of bacteremic infants than meningitic infants received this assignment, and therefore the significance of the differences in gestational age between the groups may be even greater than we report. We were unable to explore the effects of concomitant medical problems, treatments, and other environmental factors, and we relied on existing reports of feeding practices and formula testing. Clearly, additional study is needed to elucidate the lingering questions about *E. sakazakii* reservoirs, disease risk factors, and disease course.

Other gram-negative organisms, including *Escherichia coli*, *Enterobacter agglomerans*, *E. cloacae*, *Klebsiella pneumoniae*, *K. oxytoca*, and *Citrobacter freundii*, can be found in powdered infant formula (*24,25*). Powdered infant formula also has been associated with outbreaks of illness due to *Citrobacter* and multiple *Salmonella* serotypes (*13,34–38*). The degree to which *E. sakazakii* is a marker for a range of neonatal infections possibly related to powdered infant formula remains to be defined.

Certain steps can be taken immediately, however, to prevent or mitigate *E. sakazakii* disease. In a joint conference on infant formula safety in February, 2004, the World Health Organization and Food and Agriculture Organization of the United Nations made the following recommendations: 1) encourage industry partners to develop a range of affordable sterile formula options; 2) consider setting an industry standard for *Enterobacteriaecae* and *E. sakazakii* in infant formula; 3) inform infant caregivers of the risks associated with nonsterile, powdered formula; and 4) consider feeding high-risk infants sterile formula if they cannot breast-feed (*39*). The findings of our case review suggest that all neonates as well as premature infants should be included in this high-risk infant category. The American Dietetic Association has issued guidelines for infant formula preparation, storage, and administration; these should be followed by infant caregivers in hospitals and private homes (*40*). Rapid reporting of cases by clinicians could streamline data collection by local health departments and more rapidly resolve remaining questions about this illness. Manufacturer warning labels on powdered infant formula packages should stress that powdered infant formula is nonsterile and requires proper preparation, handling, and storage, and that sterile, liquid formula alternatives are available. These actions, adopted in whole or in part, may decrease the infectious risks associated with powdered formula and prevent this rare but potentially devastating disease.

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References

1. Centers for Disease Control and Prevention. *Enterobacter sakazakii* infections associated with the use of powdered infant formula—Tennessee, 2001. MMWR Morb Mortal Wkly Rep. 2002;51: 297–300.
2. Bar-Oz B, Peleg O, Block C, Arad I. *Enterobacter sakazakii* infection in the newborn. Acta Paediatr. 2001;90:356–8.
3. Biering G, Karlsson S, Clark N, Jonsdottir K, Ludvigsson P, Steingrimsson O. Three cases of neonatal meningitis caused by *Enterobacter sakazakii* in powdered milk. J Clin Microbiol. 1989;27:2054–6.

4. Block C, Peleg O, Minster N, Bar-Oz B, Simhon A, Arad I, et al. Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. Eur J Clin Microbiol Infect Dis. 2002;21:613–6.
5. Joker RN, Norholm T, Siboni KF. A case of neonatal meningitis caused by a yellow *Enterobacter*. Dan Med Bull. 1965;12:128–30.
6. Lai KK. *Enterobacter sakazakii* infections among neonates, children, and adults. Medicine. 2001;80:113–22.
7. Monroe PW, Tift WL. Bacteremia associated with *Enterobacter sakazakii* (yellow pigmented *Enterobacter cloacae*). J Clin Microbiol. 1979;10:850–1.
8. Muytjens HL, Zanen H, Sonderkamp H, Kollee L, Wachsmuth I, Farmer J. Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. J Clin Microbiol. 1983;18:115–20.
9. Van Acker J, de Smet F, Muyldermans G, Bougatef A, Naessens A, Lauwers S. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. J Clin Microbiol. 2001;39:293–7.
10. Simmons BP, Gelfand MS, Haas M, Metts L, Ferguson J. *Enterobacter sakazakii* infections in neonates associated with intrinsic contamination of a powdered milk formula. Infect Control Hosp Epidemiol. 1989;10:398–401.
11. Nazarowec-White M, Farber J. *Enterobacter sakazakii*: a review. Int J Food Microbiol. 1997;34:103–13.
12. Arseni A, Malamou-Ladas E, Koutsia C, Xanthou M, Trikka E. Outbreak of colonization of neonates with *Enterobacter sakazakii*. J Hosp Infect. 1987;9:143–50.
13. Centers for Disease Control and Prevention. *Salmonella* serotype Tennessee in powdered milk products and infant formula–Canada and United States, 1993. MMWR Morb Mortal Wkly Rep. 1993;42:516–7.
14. Coignard B, Vaillant V, Vincent JP, Leflèche A, Mariani-Kurkdjian P, Bernet C, et al. Infections sévères à *Enterobacter sakazakii* chez des nouveau-nés ayant consommé une préparation en poudre pour nourrissons, France, octobre-décembre 2004. Available from: http://www.invs.sante.fr/BEh/2006/02_03/beh_02_03_2006.pdf.
15. Urmenyi AM, Franklin AW. Neonatal death from pigmented coliform infection. Lancet. 1961;1:313–5.
16. Kleiman MB, Allen SD, Neal P, Reynolds J. Meningoencephalitis and compartmentalization of the cerebral ventricles caused by *Enterobacter sakazakii*. J Clin Microbiol. 1981;14:352–4.
17. Naqvi SH, Maxwell MA, Dunkle LM. Cefotaxime therapy of neonatal gram-negative bacillary meningitis. Pediatr Infect Dis. 1985;4:499–502.
18. Lecour H, Seara J, Miranda M. Treatment of childhood bacterial meningitis. Infection. 1989;17:343–6.
19. Noriega FR, Kotloff KL, Martin MA, Schwalbe RS. Nosocomial bacteremia caused by *Enterobacter sakazakii* and *Leuconostoc mesenteroides* resulting from extrinsic contamination of infant formula. Pediatr Infect Dis J. 1990;9:447–9.
20. Gallagher PG, Ball WS. Cerebral infarctions due to CNS infection with *Enterobacter sakazakii*. Pediatr Radiol. 1991;21:135–6.
21. Stevens JP, Eames M, Kent A, Halket S, Holt D, Harvey D. Long term outcome of neonatal meningitis. Arch Dis Child Fetal Neonatal Ed. 2003;88:F179–84.
22. Bedford H, de Louvais J, Halket S, Peckham C, Hurley R, Harvey D. Meningitis in infancy in England and Wales: follow up at age 5 years. BMJ. 2001;323:533–6.
23. Harvey D, Holt D, Bedford H. Bacterial meningitis in the newborn: a prospective study of morbidity and mortality. Semin Perinatol. 1999;23:218–25.
24. Muytjens HL, Roelofs-Willemse H, Jaspar GH. Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. J Clin Microbiol. 1988;26:743–6.
25. Iversen C, Forsythe S. Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula milk and related products. Food Microbiol. 2004;21:771–7.
26. Schuchat A, Robinson K, Wenger J, Harrison L, Farley M, Reingold A, et al. Bacterial meningitis in the United States in 1995. N Engl J Med. 1997;337:970–6.
27. Wenger JD, Hightower AW, Facklam RR, Gaventa S, Broome CV. Bacterial meningitis in the United States, 1986: report of a multistate surveillance study. J Infect Dis. 1990;162:1316–23.
28. Current marketing and use of powdered infant formula in the United States, 2003, US Food and Drug Administration. Available from http://www.fda.gov/ohrms/dockets/ac/03/briefing/3939b1_tab4c_coversheet.pdf
29. Kandhai MC, Reij MW, Gorris LG, Guillaume-Gentil O, van Schothorst M. Occurrence of *Enterobacter sakazakii* in food production environments and households. Lancet. 2004;363:39–40.
30. Hamilton JV, Lehane MD, Braig HR. Isolation of *Enterobacter sakazakii* from midgut of *Stomoxys calcitrans*. Emerg Infect Dis. 2003;9:1355–6.
31. Kuzina LV, Peloquin JJ, Vacek DC, Miller TA. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens*. Curr Microbiol. 2001;42:290–4.
32. Ongradi J. Vaginal infection by *Enterobacter sakazakii*. Sex Transm Infect. 2002;78:467–8.
33. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Munson ML. Births: final data for 2002. Natl Vital Stat Rep. 2003;52:1–113. PMID: 14717305
34. Collins RN, Treger MD, Goldsby JB, Boring JR, Coohon DB, Barr RN. Interstate outbreak of *Salmonella newbrunswick* infection traced to powdered milk. JAMA. 1968;203:838–44.
35. Thurm V, Gericke B. Identification of infant food as a vehicle in a nosocomial outbreak of *Citrobacter freundii*: epidemiological subtyping by allozyme, whole-cell protein and antibiotic resistance. J Appl Bacteriol. 1994;76:553–8.
36. Usera MA, Echeita A, Aladuena A, Blanco MC. Reymundo R, Prieto MI, et al. Interregional foodborne salmonellosis outbreak due to powdered infant formula contaminated with lactose-fermenting *Salmonella virchow*. Eur J Epidemiol. 1996;12:377–81.
37. Threlfall EJ, Ward LR, Hampton MD, Ridley AM, Rowe B, Roberts D, et al. Molecular fingerprinting defines a strain of *Salmonella enterica* serotype anatum responsible for an international outbreak associated with formula-dried milk. Epidemiol Infect. 1998;121:289–93.
38. Rowe B, Begg N, Hutchinson D, Dawkins H, Gilbert R, Jacob M, et al. *Salmonella Ealing* infections associated with consumption of infant dried milk. Lancet. 1987;2:900–3.
39. *Enterobacter sakazakii* and other microorganisms in powdered infant formula: meeting report. In: Microbiological risk assessment series, no. 6.Geneva: World Health Organization; 2004.
40. American Dietetic Association. Infant feedings: guidelines for preparation of formula and breast milk in health care facilities, 2003, Washington: American Dietetic Association; 2004.

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Venezuelan Equine Encephalitis Virus Transmission and Effect on Pathogenesis

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Quantifying the dose of an arbovirus transmitted by mosquitoes is essential for designing pathogenesis studies simulating natural infection of vertebrates. Titration of saliva collected in vitro from infected mosquitoes may not accurately estimate titers transmitted during blood feeding, and infection by needle injection may affect vertebrate pathogenesis. We compared the amount of Venezuelan equine encephalitis virus collected from the saliva of *Aedes taeniorhynchus* to the amount injected into a mouse during blood feeding. Less virus was transmitted by mosquitoes in vivo (geometric mean 11 PFU) than was found for comparable times of salivation in vitro (mean saliva titer 74 PFU). We also observed slightly lower early and late viremia titers in mice that were needle injected with 8 PFU, which represents the low end of the in vivo transmission range. No differences in survival were detected, regardless of the dose or infection route.

Designing pathogenesis studies for arboviruses that accurately simulate natural infection requires quantifying the amount of virus transmitted. Virus assays on mosquito saliva can be used to estimate the amount transmitted to vertebrates during blood feeding. However, amount of virus collected in vitro may not accurately reflect mosquito transmission.

Indirect and direct methods can be used to quantify virus delivered in mosquito saliva. Indirect methods include comparing times of death of animals exposed to a mosquito bite with those infected parenterally with known doses and comparing the time between mosquito feeding and development of viremia with the time between needle injection and development of the same viremia level. Direct methods include quantifying virus salivated into drops of blood, virus detected in vertebrate tissues immediately after mosquito feeding, virus salivated into blood agar, and virus salivated into fluids such as oil. Saliva collection in oil-filled capillary tubes, first described by Hurlbut (*1*), is widely used. Chamberlain et al. (*2*) compared several indirect and direct methods for quantifying arbovirus transmission and concluded that allowing mosquitoes to feed on serum (similar to the capillary method) is less efficient in detecting virus than other methods. Since most saliva is expectorated during probing, salivation into hanging drops or capillary tubes may be inaccurate because mosquitoes do not need to salivate to locate a blood vessel.

The amount of several arboviruses transmitted by mosquitoes has been estimated by using artificial saliva collection (*1–12*). Up to 3 $\log_{10}$ PFU of eastern equine encephalitis virus is deposited into capillary tubes filled with oil by the vector *Culiseta melanura* (*9*). Capillary collection and real-time reverse transcription (RT)–PCR estimate that *Culex pipiens pipiens* saliva contains an average of 4.3 $\log_{10}$ PFU of West Nile virus (WNV), with a range of 0.5 to 5.3 $\log_{10}$ (*8*). Recently, we estimated that the epidemic Venezuelan equine encephalitis virus (VEEV) vector, *Aedes* (*Ochlerotatus*) *taeniorhynchus*, salivates 0.2–3.2 $\log_{10}$ PFU into oil-filled capillary tubes (*12*).

Vector saliva enhances infection with many pathogens (*13–18*), and mosquito saliva is reported to enhance infection by some arboviruses. Deer and chipmunks infected with La Crosse virus by the bite of *Ae.* (*Och.*) *triseriatus* have higher and longer viremias than animals infected by needle (*19*). Mice exposed to uninfected mosquitoes and injected at the feeding site with Cache Valley virus develop enhanced viremia and seroconversion compared with unbitten mice or to those co-injected with virus and mosquito saliva (*20*). Mice have higher seroconversion rates to vesicular stomatitis virus when infected by *Ae. triseriatus*

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than by needle injection (*21*). Cytokine expression in the skin of mice infected with Sindbis virus differs after injection with mosquito salivary gland extracts than after injection with virus alone (*22*).

Other studies report no enhancement of arbovirus infection by vector saliva or feeding. Hamsters infected with WNV by mosquitoes or needle injections do not differ in level or duration of viremia, clinical manifestations, pathology, or antibody response (*23*). Birds infected with western equine encephalitis virus or Saint Louis encephalitis virus by mosquito bite or needle exhibit no difference in viremia responses (*24*), and mosquito saliva inhibits in vitro infection of dendritic cells by dengue virus (*25*).

VEEV (family *Togaviridae*, genus *Alphavirus*) is an important emerging and reemerging pathogen of humans and equines in the neotropics. Since no effective antiviral agents or a licensed human vaccine for VEEV exists, therapy is primarily supportive and prevention relies on avoidance of mosquitoes. Outbreaks of VEE can involve hundreds of thousands of equine and human cases, spread over large regions, and can last several years (*26*).

The effect of vector feeding on vertebrate infections by VEEV has not been studied. We determined the amount of VEEV in mosquito saliva collected in vitro (*12*) but did not determine whether this amount accurately reflects transmission during blood feeding. To collect saliva in a capillary tube, we need to subject the mosquito to traumatic manipulations that may affect salivation, such as anesthetization or immobilization by removal of the legs and wings. Also, mosquitoes are usually allowed to salivate into capillary tubes for a longer time (e.g., 30 minutes) than is required for engorgement on a host. Because knowing the infectious dose transmitted by mosquitoes is important for designing vertebrate infection studies, in which needles are typically used for virus delivery, we compared the amount of VEEV transmitted by mosquitoes in vitro with that transmitted in vivo. We also determined whether mosquito transmission affects viremia or time to death when compared with needle infections. Finally, we used tail amputations to investigate the extravascular or intravascular location of VEEV deposition during mosquito feeding.

Methods

Virus

We used VEEV rescued from an infectious cDNA clone derived from epidemic strain 3908 (subtype IC), a 1995 human isolate from Zulia State, Venezuela (*27*). With the exception of some IE virus strains in Mexico, subtype IC viruses are the etiologic agents of all recent VEE epidemics. Strain 3908 was passaged once in C6/36 mosquito cells before isolation of viral RNA and production of infectious cDNA. Virus recovered from baby hamster kidney cells electroporated with transcribed RNA was used for all experiments. Use of virus derived from an infectious clone minimized attenuating mutations that occur when VEEV is passaged in cell culture (*28*).

Mosquitoes

Ae. taeniorhynchus F_1 progeny of mosquitoes captured in Florida (*29*) were reared at 27°C and a relative humidity of 80% in a light:dark cycle of 12:12 hours. Adult females were infected intrathoracically with 4 $\log_{10}$ PFU of VEEV in a 1-μL volume 6–8 days after emergence and incubated at 27°C for 5 days with 10% sucrose provided ad libitum. Intrathoracic infection of mosquitoes with VEEV and incubation for 5 days generates saliva titers comparable to those that occur after oral infection (*12*).

In Vivo Transmission

Thirty-nine 6- to 8-week-old National Institutes of Health (NIH) Swiss mice (Harlan, Indianapolis, IN, USA) were anesthetized with pentobarbital, and the distal portion of the tail was exposed to 1 infected mosquito. After mosquito engorgement, the tips of the tails of 29 mice were severed and immediately homogenized in 300 μL of Eagle's minimal essential medium (MEM) supplemented with 20% fetal bovine serum (FBS) in a Mixer Mill 300 (Retsch Inc., Newton, PA, USA); the tails of 10 control mice were left intact. After centrifugation at 9,000×*g* for 5 minutes, the supernatant was removed for cell culture assays and RNA extraction with a Qiagen kit (Qiagen, Valencia, CA, USA). Vero cells were injected with 30 μL of supernatant and observed for 5 days for cytopathic effects (CPEs). All CPE-positive samples were titrated by plaque assay on Vero cells.

RNA was also extracted from the pellet of the tail homogenate with Trizol (Invitrogen, Carlsbad, CA, USA). The RNA of both supernatant and tail pellet was tested for VEEV positive-strand RNA by using real-time RT-PCR with a one-step kit (Qiagen) and a Smart Cycler (Cepheid, Sunnyvale, CA, USA). Forward (5′-CATAGTCTAGTCCGCCAAGATGTT-3′) and reverse (5′-CGATAGGGCATT GGCTGCAT-3′) primers and a probe (5′-[6-FAM] CCCGTTCCAACCAATGTAT[NFQ-MGB]-3′) were used for amplification and detection, respectively. The assay consisted of reverse transcription at 50°C for 20 minutes, denaturation at 95°C for 10 minutes, and 45 cycles at 95°C for 15 seconds, 63°C for 30 seconds, and 72°C for 30 seconds. Virus titers were extrapolated from RT-PCR results by comparison with a standard curve generated from serial dilutions of a VEEV stock quantified by plaque assay.

After a mosquito probed or fed on the mouse tail, its infection was confirmed by using forced salivation into a capillary tube as described below. All 39 mice used were kept in individual cages, monitored for signs of infection,

and bled retroorbitally 2 weeks later to test for seroconversion by using plaque-reduction neutralization tests.

To ensure that all virus injected into mouse tails could be recovered and detected, a series of known doses (2.9 $\log_{10}$, 2 $\log_{10}$, and 1 $\log_{10}$ PFU) was injected intradermally into the tail of a mouse. The tips were then severed and processed as described above. Each dose was tested in duplicate, and nearly all the injected virus was recovered (mean 2.7 $\log_{10}$ PFU recovered for 2.9 $\log_{10}$ inoculum, mean 1.9 $\log_{10}$ PFU recovered for 2.0 $\log_{10}$ inoculum, and mean 0.8 $\log_{10}$ PFU recovered for 1.0 $\log_{10}$ inoculum). Samples with known virus titers were also tested to ensure that freezing and thawing once did not alter virus content.

Saliva Assays

Thirty-nine saliva samples from intrathoracically infected mosquitoes that fed on a mouse were obtained by immobilization (legs and wings removed) and forced salivation into 10-μL capillary tubes (VWR International, West Chester, PA, USA) containing immersion oil (type B, Cargille Laboratories Inc., Cedar Grove, NJ, USA). After 30–45 minutes, salivation was confirmed by appearance of bubbles at the tip of the proboscis. An additional cohort of mosquitoes was allowed to salivate for intervals (repeated in triplicate) to duplicate times of observed mosquito feeding. The oil-saliva mixture was centrifuged into 100 μL of MEM containing 20% FBS and frozen at –80°C; 30 μL was then added to Vero cells for detection of CPE. Mosquito infection was confirmed by assaying triturated bodies and legs and wings for CPE, followed by plaque assay.

Viremia and Death

Ten 6- to 8-week-old NIH Swiss mice were infected by either 1 mosquito or intradermal injection into the ear with either 0.9 or 3.4 $\log_{10}$ PFU, which represented the range of titers injected by mosquitoes (see below). Five mice from each cohort of 10 were bled retroorbitally at 12, 24, 36, 48, 72, 96, and 120 hours postinfection, and sera were titrated by plaque assay. Mice were monitored daily until signs of encephalitis appeared, after which they were observed 4 times a day to determine time of death. The University of Texas Medical Branch Institutional Animal Care and Use Committee approved all experiments.

Statistical Analysis

Log-transformed data were normally distributed, except for data from RT-PCR assays from mouse tail homogenate pellets on which 1 mosquito probed (this group was not compared statistically). One-way analysis of variance using Tukey's test for multiple comparisons and an unpaired *t* test were used to compare all normally distributed data with GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Results

In Vivo Versus In Vitro Transmission Titers

To determine whether saliva collection accurately approximates the amount of VEEV transmitted during a mosquito bloodmeal, we quantified virus from saliva collected in vitro and virus deposited at sites of in vivo blood feeding. One mosquito feeding on the distal portion of a mouse tail transmitted a mean ± standard deviation of 1.1 ± 1.0 $\log_{10}$ PFU (11 PFU) as detected in the homogenate supernatant and 0.8 ± 0.9 $\log_{10}$ PFU (7 PFU) as estimated by real-time RT-PCR. These amounts were significantly lower ($p<0.001$) than the mean ± standard deviation amount (3.6 ± 1.5 $\log_{10}$ PFU or 4,300 PFU) deposited into capillary tubes during 30–45 minutes of salivation (Figure 1). However, the time for engorgement (<3 minutes) was much shorter than the 45 minutes allowed for in vitro salivation. Therefore, we matched times of saliva collection (range <3 minutes) to the exact engorgement times. Less VEEV ($p<0.05$) was still detected after in vivo transmission than after <3 minutes of in vitro salivation (1.9 ± 1.2 $\log_{10}$ PFU or 74 PFU).

The effect that the time of probing or feeding had on the titer of virus salivated was analyzed by timed saliva collections and mouse tail exposures. The amount of VEEV collected from mosquitoes that salivated in vitro for <3 minutes was significantly less than the amount collected from mosquitoes allowed to salivate for 45 minutes ($p<0.0001$). However, no significant difference was seen in the amount transmitted by mosquitoes allowed to completely engorge compared with mosquitoes allowed to probe only, without engorgement ($p>0.05$, 95% confidence interval –0.8 to 1.5 $\log_{10}$ PFU for the difference in the mean titers).

To address the possibility that some virus injected by feeding mosquitoes rapidly binds to or penetrates mouse cells and therefore is not measured by plaque assay, we also examined VEEV RNA content in mouse tails. No difference was detected between mean virus content in the mouse tail homogenate supernatants assayed by RT-PCR or plaques (Figure 1). Detection of relatively small amounts of viral RNA in tail homogenate pellets indicated that almost all virus remained in the supernatant and that infectious virus was not underestimated because of rapid penetration of cells or binding of virus to connective tissue (Figure 1).

Location of VEEV Deposition In Vivo

To assess intravascular versus extravascular locations of VEEV deposition by mosquitoes, we amputated the distal portions of mouse tails immediately after engorgement, and mice were observed for signs of infection. Forty percent (4/10) of control mice whose tails were not amputated after mosquito feeding survived compared with 79% (23/29) of

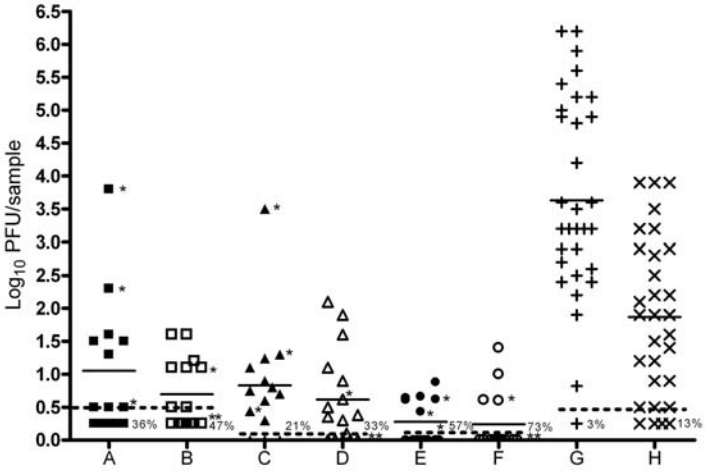

Figure 1. Titers of Venezuelan equine encephalitis virus (VEEV) transmitted in vitro or in vivo by *Aedes taeniorhynchus*. A, C, and E) Mosquitoes that engorged to completion (depicted by closed symbols). B, D, and F) Mosquitoes who probed but did not engorge (depicted by open symbols). The assay used to determine the virus titer was either cell culture assay (A and B, depicted by squares) or real-time reverse transcription–PCR (C and F) of tail homogenate supernatant (C and D, depicted as triangles) or pellet (E and F, depicted as circles). The last 2 cohorts (G and H) represent VEEV titers in saliva of mosquitoes allowed to salivate for 45 min (G, depicted as +) or for the same range of times (<3 min) required for mosquitoes to engorge completely on mouse tails, repeated in triplicate (H, depicted as ×). Solid horizontal lines indicate means, and horizontal dashed lines indicate detection limits for the assays. Symbols below the dashed lines indicate samples from infected mosquitoes (bodies and legs or wings positive for cytopathic effects) that were below the limit of detection for the assay, and numbers indicate the percentages for these negative samples (column A=36%, B=47%, C=21%, D=33%, E=57%, F=73%, G=3%, and H=13%). *Denotes mice that were bitten by a given mosquito that died.

those whose tails were amputated (p = 0.04, by Fisher exact test). No mice that survived developed neutralizing antibodies. NIH Swiss mice infected with VEEV have a death rate approaching 100%, which indicates that a systemic VEEV infection did not occur in surviving animals. This suggested that nearly all saliva and associated virus were deposited extravascularly and confined to the bite site by a lack of immediate vascular dissemination. Tail amputation nearly doubled survival rates by removing this virus before replication and dissemination.

Virus Transmitted Versus Time of Engorgement

To assess the temporal pattern of virus deposition during blood feeding, the amount of VEEV transmitted was compared with the time required for mosquito engorgement. Figure 2 shows no correlation between feeding time and amount of VEEV in mouse tails, suggesting that most virus in saliva was deposited early during probing, with minimal deposition during engorgement.

Effect on Pathogenesis in Mice Infected by Needle Versus Mosquito

To determine whether mosquito saliva affects pathogenesis of VEEV infection, mice were infected by either the bite of 1 mosquito or by intradermal needle injection. Two VEEV doses were used to represent the range of titers injected during blood feeding (Figure 1). Viremia from needle injection with a high dose did not differ from that generated by mosquito transmission (Figure 3). In contrast, viremia from a mosquito bite was higher than that from a needle injection of a low dose for the 12-hour ($p<0.05$) and 96-hour ($p<0.001$) time points. A significant difference in viremia ($p<0.001$) was also observed at the 12-hour and 96-hour time points for mice infected by needle injection of a high dose than infection of a low dose. No difference was detected in the mean survival times of mice infected by either mosquito (5.9 ± 0.6 days) or needle injection with 8 PFU (6.4 ± 0.7days) or 3.4 $\log_{10}$ PFU (6.3 ± 0.4 days) (Figure 4).

Discussion

Studies with some (*19–21*) but not all (*23*,*24*) arboviruses suggest that natural infection by mosquito bite may potentiate arboviral infection compared with parenteral infection. Because the effect of mosquito transmission on infection by VEEV has not been addressed, we assessed the infectious dose delivered by a natural vector, *Ae. taeniorhynchus*, compared with estimated doses from saliva collections. We also evaluated the effect of mosquito VEEV transmission on pathogenesis in mice.

In Vivo Versus In Vitro Transmission

Our results indicate that *Ae. taeniorhynchus* transmit less VEEV in vivo than they deposit into a capillary tube, even when saliva collection times are matched to engorgement times. We therefore caution against extended times of saliva collection in capillary tubes because mosquitoes allowed to salivate for 45 minutes expel more VEEV than those that salivate for <3 minutes, the approximate maximum time required for natural engorgement.

Our study also assessed the location of saliva deposition. As reasoned by Turell et al. (*30*,*31*), if an arbovirus were deposited intravascularly, it would quickly circulate beyond the bite site and animals with tail amputations would still become infected and die. Turell et al. reported that when the tails of suckling mice are exposed to a

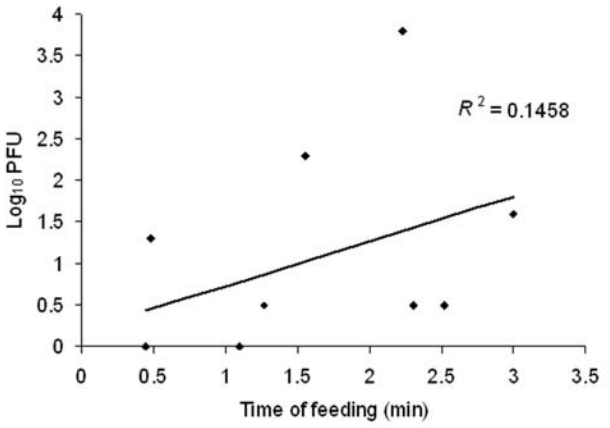

Figure 2. Amount of Venezuelan equine encephalitis virus transmitted into a mouse tail versus the time required for complete engorgement. Only samples from mosquitoes that completely engorged and transmitted detectable virus were included.

VEEV-infected *Ae. taeniorhynchus* and the tails are amputated ≤10 minutes later, 31%–37% survive compared with 4% of mice whose tails were not amputated (*30*). Our results indicating that the odds of dying are decreased by ≈50% for those whose tails were amputated suggest that saliva and VEEV are deposited both intravascularly and extravascularly. This conclusion is slightly different than that of Turell et al. (*30*,*31*), who concluded that mosquitoes inject most virus extravascularly and only small amounts intravascularly or that intravascular transmission occurs only occasionally. An explanation for the differences in death rates found in our studies and those of Turell et al. is that they used suckling mice, whereas we used adult mice. Two of our mice that had been only probed by an infected mosquito also became infected. Surprisingly, no VEEV was detected in the tail homogenate of these mice by either cell culture or RT-PCR. Because the 50% mouse subcutaneous lethal dose (LD_{50}) for VEEV strain 3908 administered in the tail is 12 PFU (D.R. Smith, unpub. data), which is greater than the LD_{50} for injection in the thigh (*12*) and an amount detectable by our methods, virus was probably deposited primarily intravascularly in these 2 animals.

Forty percent of our mice with intact tails survived after allowing an infected mosquito to engorge. NIH Swiss mice are highly susceptible to VEEV; death rates are typically 100%. Therefore, our results and those from our previous study (*12*), which reported that infected mosquitoes often deposit <12 PFU of VEEV into capillary tubes, suggest that systemically infected *Ae. taeniorhynchus* frequently transmit little or no virus. In contrast to the 40% survival rate of mice with intact tails, 100% of mice infected by mosquito bite at another site died, presumably because of a difference in the site of virus deposition. The subcutaneous LD_{50} for VEEV strain 3908 administered in the tail is 12 PFU compared with <1 PFU in the leg (D.R. Smith, unpub. data). Mosquitoes may deposit different amounts of virus at different anatomic sites, depending on accessibility of blood vessels.

Time of Engorgement and Infectious Dose Transmitted

The amount of VEEV transmitted by *Ae. taeniorhynchus* did not correlate with time of engorgement. However, we did not count how many times the mosquito probed before beginning to engorge. Assuming that most mosquito saliva is injected during the intradermal probing period that precedes cannulation of a blood vessel and that infection of the host correlates with the duration of salivation during probing, probing frequency could affect transmission outcome and should be investigated.

Effects on VEE Pathogenesis of Infection by Needle Versus Mosquito

Because mosquitoes transmit a wide range of arbovirus doses, we injected mice with 2 doses that represented the range of VEEV transmitted in vivo. No difference in viremia was detected between mice infected by a mosquito than by needle injection of a high dose. However, mice infected by mosquito bite showed higher viremia titers at the early (12 hours) and late (96 hours) time points than did mice infected with a low dose given by needle. Because mice injected with the high dose also had higher viremia titers at some time points than did mice in the low-dose

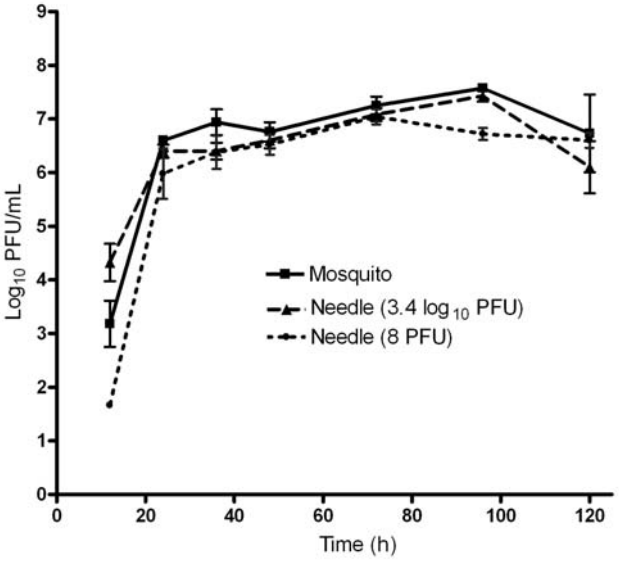

Figure 3. Viremia in mice infected by 1 mosquito bite or intradermally by needle injection with 2 different doses of Venezuelan equine encephalitis virus representing the range of doses delivered during blood feeding (Figure 1). Five animals per cohort were bled at each time point. Error bars indicate standard deviations.

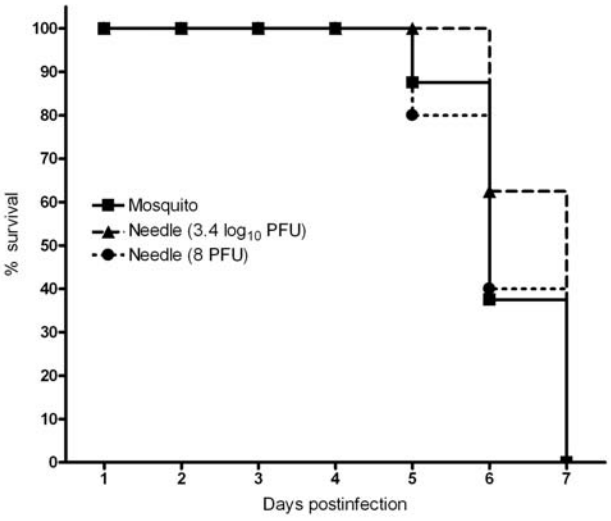

Figure 4. Survival of cohorts of 10 mice infected with Venezuelan equine encephalitis virus either by 1 mosquito bite or by intradermal needle injection with 2 doses representing the range of virus titers delivered during mosquito blood feeding (Figure 1).

cohort (Figure 3), the difference in the infection by mosquito bite versus a low-dose injection by needle may indicate only that some mosquitoes transmitted doses >8 PFU. The only way to confirm this slight effect of mosquito transmission on early and late viremia would be to duplicate the exact distribution of in vivo transmission titers by needle injections. However, volumes injected by mosquitoes compared with those injected by needles would differ, as would intradermal sites of deposition. Another approach is to coinject mosquito saliva with virus (*20*,*22*), but the same volume and site discrepancies would apply.

Although route (mosquito versus needle) or dose of VEEV had no detectable effect on death rates (Figure 4), mosquito transmission enhancement of early viremia titers could affect subsequent vector infection, and comparable studies with natural reservoir or amplification hosts are needed to assess this possibility. In preliminary studies, no difference in the viremia response of spiny rats was observed after VEEV infection by needle compared with mosquito bites (A.S. Carrara and S.C. Weaver, unpub. data).

Our results agree with reports of little or no enhancement of alphaviral infections by mosquito transmission (*24*). In several studies describing enhancement of arboviral infection by mosquito transmission, multiple mosquitoes were allowed to feed and transmit to 1 vertebrate (*19–21*), or salivary gland extracts from many mosquitoes were injected with virus (*20*,*22*). Because natural infection rates of mosquitoes are typically low, simultaneous transmission by >1 vector is probably rare. In addition, virus amounts injected by needle in these studies may have been less than the virus amount transmitted by mosquitoes, which would confound interpretation. Artificial conditions used for several experiments demonstrating potentiation of arbovirus infection by mosquito transmission may therefore exaggerate the true effect.

Significance for Pathogenesis Studies

In conclusion, *Ae. taeniorhynchus* transmit less VEEV in vivo than they deposit in vitro into capillary tubes. Mosquito transmission of VEEV has little or no effect on the overall murine viremia profile and none on death. To design VEE pathogenesis studies that simulate natural infection, a dose range from 10 PFU to 1,000 PFU is recommended to simulate mosquitoborne infections. Because VEEV saliva titers differ among mosquito species (*12*), comparable studies should be conducted with other vectors.

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Ms Smith is a graduate student at the University of Texas Medical Branch in Galveston. Her research interests include mechanisms of arbovirus transmission by mosquitoes and the pathogenesis of VEEV in both the mosquito vectors and vertebrate hosts.

References

1. Hurlbut HS. Mosquito salivation and virus transmission. Am J Trop Med Hyg. 1966;15:989–93.
2. Chamberlain RW, Kissling RE, Sikes RK. Studies on the North American arthropod-borne encephalitides. VII. Estimation of amount of eastern equine encephalitis virus inoculated by infected *Aedes aegypti*. Am J Hyg. 1954;60:286–91.
3. Aitken TH. An in vitro feeding technique for artificially demonstrating virus transmission by mosquitoes. Mosquito News. 1977;37:130–3.
4. Collins WE. Transmission of Semliki Forest virus by *Anopheles albimanus* using membrane feeding techniques. Mosquito News. 1963;23:96–9.
5. Gubler DJ, Rosen L. A simple technique for demonstrating transmission of dengue virus by mosquitoes without the use of vertebrate hosts. Am J Trop Med Hyg. 1976;25:146–50.
6. Mellink JJ. Transmission of Venezuelan equine encephalomyelitis virus by *Aedes aegypti* (Diptera: Culicidae) to mice previously exposed to vector antigens. J Med Entomol. 1982;19:371–5.
7. Ross RW. A laboratory technique for studying the insect transmission of animal viruses, employing a bat-wing membrane, demonstrated with two African viruses. J Hyg (Lond). 1956;54:192–200.

8. Vanlandingham DL, Schneider BS, Klingler K, Fair J, Beasley D, Huang J, et al. Real-time reverse transcriptase-polymerase chain reaction quantification of West Nile virus transmitted by *Culex pipiens quinquefasciatus*. Am J Trop Med Hyg. 2004;71:120–3.
9. Weaver SC, Scott TW, Lorenz LH. Patterns of eastern equine encephalomyelitis virus infection in *Culiseta melanura* (Diptera: Culicidae). J Med Entomol. 1990;27:878–91.
10. Ross RW. Transmission experiments with chikungunya A and B virus. Experiments using a new apparatus. Entebbe, Uganda: Virus Research Institute; 1953. p. 13–14.
11. Davis NC. Attempts to determine the amount of yellow fever virus injected by the bite of a single infected *Stegomyia* mosquito. American Journal of Tropical Medicine. 1934;14:343–54.
12. Smith DR, Carrara A, Aguilar P, Weaver SC. Evaluation of methods to assess transmission potential of Venezuelan equine encephalitis virus by mosquitoes and estimation of mosquito saliva titers. Am J Trop Med Hyg. 2005;73:33–9.
13. Titus RG, Ribeiro JM. Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. Science. 1988;239:1306–8.
14. Shaw MK, Tilney LG, McKeever DJ. Tick salivary gland extract and interleukin-2 stimulation enhance susceptibility of lymphocytes to infection by *Theileria parva* sporozoites. Infect Immun. 1993;61:1486–95.
15. Ribeiro JM. Role of saliva in blood-feeding by arthropods. Annu Rev Entomol. 1987;32:463–78.
16. Nuttall PA, Jones LD, Labuda M, Kaufman WR. Adaptations of arboviruses to ticks. J Med Entomol. 1994;31:1–9.
17. Titus RG, Ribeiro JM. The role of vector saliva in transmission of arthropod-borne disease. Parasitol Today. 1990;6:157–60.
18. Theodos CM, Titus RG. Salivary gland material from the sand fly *Lutzomyia longipalpis* has an inhibitory effect on macrophage function in vitro. Parasite Immunol. 1993;15:481–7.
19. Osorio JE, Godsey MS, Defoliart GR, Yuill TM. La Crosse viremias in white-tailed deer and chipmunks exposed by injection or mosquito bite. Am J Trop Med Hyg. 1996;54:338–42.
20. Edwards JF, Higgs S, Beaty BJ. Mosquito feeding-induced enhancement of Cache Valley virus (*Bunyaviridae*) infection in mice. J Med Entomol. 1998;35:261–5.
21. Limesand KH, Higgs S, Pearson LD, Beaty BJ. Potentiation of vesicular stomatitis New Jersey virus infection in mice by mosquito saliva. Parasite Immunol. 2000;22:461–7.
22. Schneider BS, Soong L, Zeidner NS, Higgs S. *Aedes aegypti* salivary gland extracts modulate anti-viral and TH1/TH2 cytokine responses to Sindbis virus infection. Viral Immunol. 2004;17:565–73.
23. Sbrana E, Tonry JH, Xiao SY, da Rosa AP, Higgs S, Tesh RB. Oral transmission of West Nile virus in a hamster model. Am J Trop Med Hyg. 2005;72:325–9.
24. Reisen WK, Chiles RE, Kramer LD, Martinez VM, Eldridge BF. Method of infection does not alter response of chicks and house finches to western equine encephalomyelitis and St. Louis encephalitis viruses. J Med Entomol. 2000;37:250–8.
25. Ader DB, Celluzzi C, Bisbing J, Gilmore L, Gunther V, Peachman KK, et al. Modulation of dengue virus infection of dendritic cells by *Aedes aegypti* saliva. Viral Immunol. 2004;17:252–65.
26. Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC. Venezuelan equine encephalitis. Annu Rev Entomol. 2004;49:141–74.
27. Weaver SC, Salas R, Rico-Hesse R, Ludwig GV, Oberste MS, Boshell J, et al. Re-emergence of epidemic Venezuelan equine encephalomyelitis in South America. VEE Study Group. Lancet. 1996;348:436–40.
28. Bernard KA, Klimstra WB, Johnston RE. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology. 2000;276:93–103.
29. Coffey LL, Weaver SC. Susceptibility of *Ochlerotatus taeniorhynchus* and *Culex nigripalpus* for Everglades virus. Am J Trop Med Hyg. 2005;73:11–6.
30. Turell MJ, Spielman A. Nonvascular delivery of Rift Valley fever virus by infected mosquitoes. Am J Trop Med Hyg. 1992;47:190–4.
31. Turell MJ, Tammariello RF, Spielman A. Nonvascular delivery of St. Louis encephalitis and Venezuelan equine encephalitis viruses by infected mosquitoes (Diptera: Culicidae) feeding on a vertebrate host. J Med Entomol. 1995;32:563–8.

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Bat-transmitted Human Rabies Outbreaks, Brazilian Amazon

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We describe 2 bat-transmitted outbreaks in remote, rural areas of Portel and Viseu Municipalities, Pará State, northern Brazil. Central nervous system specimens were taken after patients' deaths and underwent immunofluorescent assay and histopathologic examination for rabies antigens; also, specimens were injected intracerebrally into suckling mice in an attempt to isolate the virus. Strains obtained were antigenically and genetically characterized. Twenty-one persons died due to paralytic rabies in the 2 municipalities. Ten rabies virus strains were isolated from human specimens; 2 other cases were diagnosed by histopathologic examination. Isolates were antigenically characterized as *Desmodus rotundus* variant 3 (AgV3). DNA sequencing of 6 strains showed that they were genetically close to *D. rotundus*–related strains isolated in Brazil. The genetic results were similar to those obtained by using monoclonal antibodies and support the conclusion that the isolates studied belong to the same rabies cycle, the virus variants found in the vampire bat *D. rotundus*.

Rabies virus, the prototype species in the family *Rhabdoviridae*, is a single-stranded, RNA, negative-sense, nonsegmented virus of the genus *Lyssavirus*; it is the causal agent of rabies, a disease that has been poorly studied in most developing countries (*1*,*2*). Nonetheless, Brazil has implemented rabies control measures, and urban human rabies, transmitted by dogs and cats, has decreased from 73 cases in 1990 to 17 cases in 2003 (*3*). Carnivores and bats are the primary reservoirs of rabies virus in all continents, and bat-transmitted rabies is relatively commonly diagnosed in Latin American and Caribbean countries (*1*,*4*–*6*). In Brazil, a small number of human cases have been confirmed as having been transmitted by vampire bat bites (*7*,*8*), but because surveillance has improved in the last few years, the occurrence of sporadic episodes suggests a situation similar to that observed in other American countries (*5*,*9*,*10*). Indeed, ≈39 cases of human rabies have been reported in the United States since the 1950s, and cases of rabies transmitted by vampire bat bites are commonly reported in Mexico, Chile, Colombia, Peru, Venezuela, and other New World countries (*1*,*5*,*6*,*9*,*11*,*12*).

Although rabies control measures have improved in many South American countries, the transmission of the disease by bats has increased and has become a public health concern, and several human cases have been detected. Outbreaks of bat-transmitted rabies have occurred in several remote areas in Peru (*5*,*13*), Venezuela (*9*), and more recently in Brazil (*7*). This article reports the results of an epidemiologic investigation and the antigenic and genetic characterization of rabies virus isolated during outbreaks of the bat-transmitted disease that occurred in March and May 2004 in remote areas of Portel and Viseu municipalities, respectively, in Pará State, Brazilian Amazon region.

Material and Methods

Patients

All patients reported receiving vampire bat bites several weeks or months before manifesting encephalitic symptoms. All were poor persons who lived in primitive conditions in the Acuty-Perera River communities in

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Portel or in the Curupati community in the municipality of Viseu, both in Pará State, Brazilian Amazon region (Figure 1). All patients exhibited a similar disease pattern, characterized by acute ascendant paralytic encephalitis. Twelve patients were hospitalized and after their deaths, an autopsy was performed and central nervous system (CNS) specimens were obtained for laboratory diagnostic procedures. Patient ages ranged from 2 to 58 years. Detailed information concerning the patients is shown in the Table.

Figure 1. Pará State showing the municipalities of Portel and Viseu, where vampire bat–transmitted rabies cases were reported. Source: Adapted from Government of Pará State website: www.brasilrepublica.hpg.ig.com.br/para.htm

Areas of Occurrence

Many persons live in poor conditions in both municipalities where cases were reported. The municipality of Portel (50°57′W, 1°59′S), is situated in the Marajó Island region, state of Pará, and is ≈278 km distant from Belém, the state capital; access to Belém is by the Amazon River. Portel has ≈41,500 inhabitants (1.6 inhabitants/km^2); ≈55% of them live in rural areas. The principal economic activities are wood extraction agriculture (especially cultivation of manioc), and hook-and-line fishing; cattle grazing is uncommon. In the Acuty-Perera River area, where cases were reported, the infected persons were from the following 3 communities: Ajará, Laranjal (Aparecida), and Tauaçu.

The municipality of Viseu, (50°49′W, 1°56′S) is in the Bragantina region of the state of Pará, 320 km distant from Belém, with access by highways. It has ≈52,893 inhabitants (10.2 inhabitants/km^2); 68% live in rural areas. Rabies cases were reported in the Curupati community where ≈77 families lived. Economic activities include cultivation of manioc and hook-and-line fishing.

Virus Isolation

All laboratory analyses were performed under pressurized containment cabinets, class II B2. From all patient specimens, homogenates were obtained as previously described (*14*). Briefly, 0.02 mL of each suspension in phosphate-buffered saline (pH 7.4) containing fraction V bovine albumin solution (0.75%), penicillin (100 IU/mL), and streptomycin (100 μg/mL), was injected intracerebrally into 12 newborn mice. After injection, the mice were observed daily for 3 weeks or until the animals became sick, when their brains were removed and used for immunofluorescence assay (IFA) or stored at –70°C for further molecular biology procedures.

Detection and Characterization of Isolates

All human CNS samples and suckling mouse brains were used to prepare impression smears, which were examined by direct IFA with a fluorescent antirabies conjugate, as described elsewhere (*15*). All rabies virus strains isolated were antigenically typed by indirect IFA by using a panel of 8 monoclonal antibodies prepared against the viral nucleoprotein (Centers for Disease Control and Prevention, Atlanta, GA, USA) (*9*,*16*). Six original CNS samples (3066M, 3067M, 3068M, 3072M, and 3522M from Portel and 5214 from Viseu) were tested in a reverse transcription–PCR that amplifies a 1,352-bp fragment of the nucleoprotein gene with sense primer N1 5′-ATGGATGCCGACAAGATT 3′and anti-sense primer N2 5′-TTATGAGTCACTCGAATA 3′ as described by Carnieli et al. (*17*) by using Superscript II Reverse Transcriptase (Invitrogen Ltd., São Paulo, Brazil) and Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

PCR products were excised from agarose gel, purified with QIAquick gel extraction kit (Qiagen Inc., Valencia, CA, USA), and sequenced reaction with anti-sense primer and DYEnamic ET Dye Terminator (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's instructions, in 4 replicates. The sequences were resolved in a MegaBACE DNA sequencer (Amersham Biosciences).

The final sequence of each strain was aligned by the Clustal method with Bioedit (*18*) (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and MEGA 2.1 (www.megasoftware.net), with homologous sequences derived from GenBank (accession numbers shown in Figure 2). The alignment was used to build a neighbor-joining distance tree with the Kimura 2-parameter model and 1,000 bootstrap replicates with MEGA 2.1 (*19*).

Table. Generic information regarding 12 patients with diagnosis of paralytic rabies from Portel and Viseu municipalities, Pará State

Patient	Age (y), sex	Place	Municipality	Virus isolation	Onset	Death	Place of bite(s)	GenBank accession no.*
1	11, F	Tauaçu	Portel	Yes	19 Mar	28 Mar	Feet	NA
2	22, F	Ajará	Portel	Yes	9 Mar	24 Mar	Feet	DQ097077
3	42, M	Ajará	Portel	Yes	10 Mar	31 Mar	Feet	NA
4	30, M	Ajará	Portel	Yes	19 Mar	30 Mar	Feet	NA
5	12, M	Ajará	Portel	Yes	9 mar	30 Mar	Feet	DQ097076
6	26, F	Ajará	Portel	Yes	19 Mar	1 Apr	Feet	DQ097080
7	30, F	Laranjal	Portel	Yes	23 Mar	3 Apr	Feet	DQ097078
8	2, M	Laranjal	Portel	Yes	1 Apr	6 Apr	Head	DQ097079
9	22, M	Laranjal	Portel	No†	15 Mar	28 Mar	Feet	NA
10	2, M	Laranjal	Portel	No†	16 Mar	30 Mar	Head/feet	NA
11	58, M	Curupati	Viseu	Yes	30 Apr	17 May	NA	DQ097075
12	22, M	Curupati	Viseu	Yes	5 May	14 May	NA	NA

*NA, not available or unknown.
†Diagnosis by histopathologic examination.

Results

Twenty-one cases of bat-transmitted rabies were reported (15 from Portel and 6 from Viseu). Twelve were confirmed by laboratory diagnostic procedures, and 9 cases (5 of Portel and 4 of Viseu) were confirmed by clinical and epidemiologic linkage. Of the laboratory-confirmed cases, 2 from Portel were diagnosed by histopathologic examination and 10 (8 from Portel and 2 from Viseu) were diagnosed by IFA or by virus isolation in suckling mice. Antigenic characterization showed variant 3 (AgV3), the primary reservoir of which is the vampire bat *Desmodus rotundus*. One additional rabies virus isolate obtained from a *D. rotundus* was also typed as AgV3 (E.S.T. da Rosa and P.F.C. Vasconcelos, unpub. data).

The nucleotide identity among the 6 sequences from the human rabies strains of this study was 99.3%; identity among the 5 strains from Portel was 100%, and identity among these and the strain from Viseu was 97.2%. These same 6 strains had an identity of 97% when compared to AgV3 *D. rotundus* strains and 82.8% when compared to AgV2 Brazilian dog strains. Furthermore, the nucleotide identity among the 6 human strains and strains related to raccoons was 85.2%; it was 84.5% when compared to fixed strains CVS and AV01 (GenBank accession nos. D42112.1 and X13357.1, respectively). The phylogenetic tree (Figure 2) shows a clustering pattern that is in accordance with each specific host or variant of the rabies virus, each cluster supported by a bootstrap value of at least 98%.

The 6 human isolates were genetically grouped in the *D. rotundus* cluster, supported by a bootstrap value as high as 1,000. The 5 strains from Portel grouped together in an exclusive polytomic subcluster, supported by a bootstrap value of 97%, while strain 5214M from Viseu grouped in a paraphyletic and more resolved subcluster among samples detected in *D. rotundus* and *Artibeus* spp. from Brazil and an AgV3 strain detected in a Brazilian cat (AY563517.1).

Discussion

This is the first outbreak of vampire bat–transmitted rabies reported in Brazil in which rabies virus was isolated from humans and bats and in which the isolated strains were antigenically and genetically characterized. Previous reports of bat-transmitted outbreaks were based only on clinical and epidemiologic linkage, and in all of these outbreaks, infected persons were living in small, remote areas with difficult access in the Amazon region, including isolated Indian villages and clandestine gold mining areas in the states of central and northern Brazil. In these episodes, deaths were reported several weeks after the patients had died and therefore, no clinical specimens could be examined (*7*,*20*).

In contrast, although the outbreaks of Portel and Viseu occurred in isolated remote areas, access to them was facilitated by rivers (Portel) and highways (Viseu) and also by

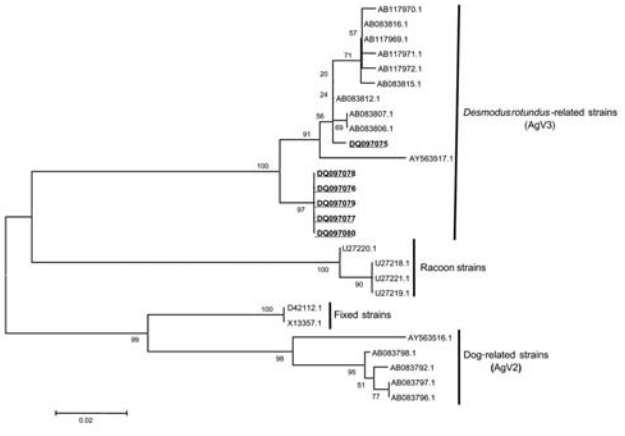

Figure 2. Neighbor-joining tree with K2P model based on partial nucleoprotein gene sequences of rabies virus from *Desmodus rotundus* AgV3, raccoons, fixed strains, and dog AgV2. Each taxon is represented by its respective GenBank accession number (human strains from the present study are in bold and underlined). Numbers at each node are 1,000 bootstrap replicate values; the bar indicates genetic distance.

the fact that both areas were considerably closer to Belém, the capital of Pará State.

Moreover, in the last few years, improvement in the surveillance for rabies and several other less-studied infectious diseases by the Brazilian Ministry of Health and Secretary of Health for Pará State has resulted in a more sensitive system for detecting and investigating all relevant and unusual episodes suspected to be bat-transmitted rabies. The number of cases of bat-transmitted human rabies is, therefore, expected to increase: not only because of the improved epidemiologic surveillance system but also because of the reduction in opportunities for the bat's life cycle to be maintained in urban areas (*1,5,7–9,12*). An epidemiologic investigation of cases in humans in Portel and Viseu areas showed that vampire bat bites are common. Indeed, all victims had a history of bat bites, but bites were not considered a risk for acquiring rabies. Moreover, 1,558 persons in Portel (Acuty Perera River communities) reported ≥1 episode of vampire bat aggression, and many inhabitants reported several bat bites in the 12 months before the outbreak. All these persons received postexposure treatment (antirabies serum and 5 doses of diploid human cells vaccine). Persons reporting bat bites for >1 year (838 persons in the Acuty Perera River communities) received preexposure treatment (3 doses of diploid human cells vaccine). After treatment measures, cases of rabies by bat aggression were no longer reported. Moreover, in Portel County, 4,504 dogs and 1,789 cats were also vaccinated (*3*).

Nonetheless, the lack of clinicians to diagnose the first cases at local hospitals has contributed to the increased number of noninvestigated cases and for the delay in recognizing them as rabies. In Peru, the country with the highest prevalence of bat-transmitted rabies in the Americas, several epidemics have been recognized. Delay in recognizing the disease was associated with several outbreaks (*5,13*). In Portel and Viseu, all clinical cases were characterized as ascendant paralytic rabies. Patients exhibited paresis, paralysis, dyspnea and difficulties of speech, soreness or lethargy, photophobia, aerophobia, hydrophobia, and coma, symptoms similar to those previously reported in other vampire bat–transmitted rabies outbreaks (*1,5,7,21–24*). The topology of the neighbor-joining tree, shown in Figure 2, grouped all rabies strains according to each respective host and variant, which validates the sequenced region and the tree-building method; in addition, the nucleotide identity among AgV3 strains and the human strains studied here (97%) match the thresholds described for different variant-host associations (*25*). The distance-based phylogenetic analysis of the N gene, based on full-length (1,350 nt) or partial (200–300 nt) sequencing, allows highly statistically supported clusterings for each rabies virus variant or each host-specific variant; the method is the most efficient in rabies molecular epidemiology (*26*).

These results lead to the conclusion that all strains of rabies virus isolated from humans during the rabies outbreak in northern Brazil in 2004 are related to the *D. rotundus* variant commonly found in Brazil, which supports the data generated by antigenic typing. Nevertheless, since all 5 sequenced strains from Portel grouped in different subclusters when compared with the sequenced strain from Viseu, this finding might be a sign that regional patterns of lineages of AgV3 rabies virus exist. Because the 2 municipalities are 530 km apart and in different ecologic and geographic regions, the Portel subclusters represent a unique and exclusive lineage. Whether the strain involved in the Viseu outbreak is in fact, monophyletic with all *D. rotundu*s strains used in the phylogenetic analysis or could give rise to other paraphyletic subclusters still remains to be answered by analysis of more samples from the same area.

The complete identity among the 5 Portel strains might be due to the high attack rates reported in this specific outbreak, because the same virus lineage or subclusters would be rapidly transmitted by a homogeneously infected population of vampire bats sharing an exclusive lineage of rabies virus. Subclusters in AgV3 from *D. rotundus* are not an uncommon binding, and strains from the same area are likely to cluster together (*27*). In Latin America, area-specific clusterings have been described for AgV3 in Argentina, Venezuela, and Mexico, where clusterings divergent from those in Brazil are known to occur (*6,9,12,28*).

The genetic data obtained from the human strains studied here might be used to follow in a more accurate way, the population of *D. rotundus* involved in transmission when the rabies virus strains detected in these bats and regional ecologic information about these become available. This information might provide a powerful tool to help understand the factors that facilitated the outbreak and prevent others in the future.

The isolation of a strain from the vampire bat *D. rotundus* in Breves, a municipality bordering Portel (Figure 1) and close to where human infections were reported, is definitively incriminates this species in the transmission of rabies virus in Pará State (E.S.T. da Rosa and P.F.C. Vasconcelos, unpub. data).

Previous reports showed a rabid infection frequency ranging from 0% to 3% for *D. rotundus*, which is associated with high or low endemicity (*29*). In the outbreaks described in this article, rabies virus was only isolated near the Portel area (Breves municipality) from a single *D. rotundus* among 23 of 132 bats studied. The aggression of vampire bats in these remote areas may be because persons live in unprotected dwellings (houses either without walls or without windows and doors) as shown in Figure 3, and the number of wild animals, cattle or equines, is small.

Moreover, in June 2005, 9 other vampire bat–transmitted rabies cases in humans were reported in communities of Augusto Correa Municipality (Figure 1), which borders Viseu (E.S. Travassos da Rosa and P.F.C. Vasconcelos, unpub. data).

Finally, public health campaigns should carried out to alert inhabitants of remote, small communities in the Amazon region to the risk of bat bites in transmitting rabies, and the need for all persons who report attacks of bats to undergo postexposure treatment to prevent other cases of vampire bat–transmitted rabies. Also, ecologic studies should be initiated to clarify the dynamics of rabies infection between populations of *D. rotundus* in affected areas.

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Figure 3. Typical human dwellings in the Acuty Perera River region, Portel (A), and in the Curupati area, Viseu (B). Note in (A) a house without walls on the Acuty Perera River.

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References

1. Rupprecht CE, Hanlon CA, Hamachudha T. Rabies re-examined. Lancet Infect Dis. 2002;2:327–43.
2. Warrell MJ, Warrell DA. Rabies and other lyssavirus diseases. Lancet. 2004;363:959–69.
3. Ministério da Saúde. Surto de raiva human transmitida por morcegos no município de Portel-Pará, Março/Abril de 2004. Boletim Eletrônico Epidemiológico (Brasília). 2004;4:2–5.
4. Diaz AM, Papo S, Rodriguez A, Smith JS. Antigenic analysis of rabies-virus isolates from Latin America and the Caribbean. Zentralbl Veterinarmed B. 1994;41:153–60.
5. Warner CK, Zaki SR, Shieh W-J, Whitfield SG, Smith JS, Orciari LA, et al. Laboratory investigation of human deaths from vampire bat rabies in Peru. Am J Trop Med Hyg. 1999;60:502–7.
6. Velasco-Villa A, Gomez-Sierra M, Hernandez-Rodriguez G, Juarez-Islas V, Melendez-Felix A, Vargas-Pino F, et al. Antigenic diversity and distribution of rabies virus in Mexico. J Clin Microbiol. 2002;40:951–8.
7. Schneider MC, Santos-Burgoa C, Aron J, Munoz B, Ruiz-Velazco S, Uieda W. Potential force of infection of human rabies transmitted by vampire bats in the Amazonian region of Brazil. Am J Trop Med Hyg. 1996;55:680–4.
8. Schneider MC, Aron J, Santos-Burgoa C, Uieda W, Ruiz-Velazco S. Common vampire bat attacks on humans in a village of the Amazon region of Brazil. Cad Saude Publica. 2001;17:1531–6.
9. De Mattos CC, De Mattos CA, Loza-Rubio E, Aguilar-Setién A, Orciari LA, Smith JS. Molecular characterization of rabies virus isolates from Mexico: implications for transmission dynamics and human risk. Am J Trop Med Hyg. 1999;61:587–97.
10. Rohde RE, Mayes BC, Smith JS, Neill SU. Bat rabies, Texas, 1996–2000. Emerg Infect Dis. 2004;10:948–52.
11. Morimoto K, Patel M, Corisdeo S, Hooper Dc, Fu ZF, Rupprecht CE, Characterization of a unique variant of bat rabies virus responsible for newly emerging human cases in North America. Proc Natl Acad Sci U S A. 1996;93:5653–8.
12. de Mattos CA, de Mattos CC, Smith JS, Miller ET, Papo S, Utrera A, et al. Genetic characterization of rabies field isolates from Venezuela. J Clin Microbiol. 1996;34:1553–8.
13. Lopez A, Mirand P, Tejada E, Fishbein DB. Outbreak of human rabies in the Peruvian jungle. Lancet. 1992;339:408–11.
14. Koprowski H. The mouse inoculation test. In: Meslin FX, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies. Geneva: World Health Organization; 1996. p. 80–7.
15. Dean DJ, Abelseth MK. The fluorescent antibody test. In: Kaplan MM, Koprowski E, editors. Laboratory techniques in rabies, 3rd edition. Geneva: World Health Organization; 1973. p. 73–84.
16. Favoretto SR, Carrieri ML, Cunha EM, Aquiar EA, Silva LH, Sodre MM, et al. Antigenic typing of Brazilian rabies virus samples isolated from animals and humans, 1989-2000. Rev Inst Med Trop Sao Paulo. 2002;44:91–5.

17. Carnieli Jr. Brandão PE, Macedo CI, Castilho JG, Zanetti CR, Carrieri ML, et al. Rabies in cats caused by rabies virus variants of bats: a new challenge to public health. In: The XV International Conference of Rabies in the Americas, 2004, Santo Domingo. Santo Domingo, Dominican Republic: Pan American Health Organization; 2004. pp. 37–8.
18. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser. 1999;41:95–8.
19. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: Molecular evolutionary genetics analysis software. Tempe (AZ): Arizona State University; 2001.
20. Mayen F. Haematophagous bats in Brazil, their role in rabies transmission, impact on public health, livestock industry and alternatives to an indiscriminate reduction of bat population. J Vet Med. 2003;B50:469–72.
21. Hurst EW, Pawan JL. An outbreak of rabies in Trinidad without history of bites and with symptoms of acute ascending paralysis. Lancet. 1931;ii:622–5.
22. Hurst EW, Pawan JL. A further account of the Trinidad outbreak of acute rabies myelitis. J Pathol Bacteriol. 1932;35:301–21.
23. Pawan JL. Rabies in the vampire bat of Trinidad, with special reference to the clinical course and the latency of infection. Ann Trop Med Parasitol. 1936;30:101–29.
24. Nehaul BBG. Rabies transmitted by bats in British Guiana. Am J Trop Med Hyg. 1955;4:550–3.
25. Smith JS, Orciari LA, Yager PA, Seidel HD, Warner CK. Epidemiologic and historical relationships among 87 rabies virus isolates as determined by limited sequence analysis. J Infect Dis. 1992;166:296–307.
26. Smith JS. Molecular epidemiology. In: Jackson AC, Wunner WH, editors. Rabies. San Diego: Academic Press; 2002. p. 79–111.
27. Ito M, Arai YT, Itou T, Sakai T, Ito, FH,Takasaki T, et al. Genetic characterization and geographic distribution of rabies virus isolates in Brazil: identification of two reservoirs, dogs and vampire bats. Virology. 2001;284:214–22.
28. Cisterna D, Bonaventura R, Caillou S, Pozp O, Andreau ML, Fontana LD, et al. Antigenic and molecular characterization of rabies virus in Argentina. Virus Res. 2005;109:139–47.
29. Côrtes VA, Souza LC, Uieda W, Figueiredo AC. Abrigos diurnos e infecção rábica em morcegos de Botucatu, São Paulo, Brasil. Vet e Zoot São Paulo. 1994;6:179–86.

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ANOTHER DIMENSION

Grandmother Speaks of the Old Country

Lola Haskins

That year there were many deaths in the village.
Germs flew like angels from one house to the next
and every family gave up its own. Mothers
died at their mending. Children fell at school.
Of three hundred twenty, there were eleven left.
Then, quietly, the sun set on a day when no one
died. And the angels whispered among themselves.
And that evening, as he sat on the stone steps,
your grandfather felt a small wind on his neck
when all the trees were still. And he would tell us
always, how he had felt that night, on the skin
of his own neck, the angels, passing.

Streptococcus suis Sequence Type 7 Outbreak, Sichuan, China

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An outbreak of *Streptococcus suis* serotype 2 emerged in the summer of 2005 in Sichuan Province, and sporadic infections occurred in 4 additional provinces of China. In total, 99 *S. suis* strains were isolated and analyzed in this study: 88 isolates from human patients and 11 from diseased pigs. We defined 98 of 99 isolates as pulse type I by using pulsed-field gel electrophoresis analysis of *Sma*I-digested chromosomal DNA. Furthermore, multilocus sequence typing classified 97 of 98 members of the pulse type I in the same sequence type (ST), ST-7. Isolates of ST-7 were more toxic to peripheral blood mononuclear cells than ST-1 strains. *S. suis* ST-7, the causative agent, was a single-locus variant of ST-1 with increased virulence. These findings strongly suggest that ST-7 is an emerging, highly virulent *S. suis* clone that caused the largest *S. suis* outbreak ever described.

Streptococcus suis, a swine pathogen, is increasing in clinical importance in countries with intensive swine industries (*1*–*4*). Infection in humans is considered an occupational disease that affects persons who work in close contact with pigs or pork byproducts (*5*). Currently, 35 capsular types or serotypes are officially described; *S. suis* serotype 2 is considered the most prevalent and virulent in pigs and humans (*6*). The first human case was recorded in 1968, and only 200 cases have been subsequently reported globally through June 2005 (*5*). In some Asian countries, *S. suis* may be the second most common cause of adult streptococcal meningitis (*7*).

However, a larger outbreak due to *S. suis* serotype 2 emerged in the summer of 2005 in Sichuan Province, China. In total, 215 cases were reported; 38 deaths occurred in 202 villages in 36 counties. New cases were identified in 4 additional provinces in China after the Sichuan outbreak. A striking feature of this outbreak was the unusually high rate of death and streptococcal toxic shocklike syndrome (STSS) as a clinical manifestation. Indeed, reported symptoms included high fever, malaise, nausea, vomiting, and diarrhea, followed by meningitis, subcutaneous hemorrhage, toxic shock, and coma in severe cases. This increased severity of *S. suis* infections in humans, such as the shorter incubation time, more rapid disease progression, and higher death rate, underscores the need to better understand the factors associated with *S. suis* infection.

The aim of this study was to characterize and analyze the causative agent of this unusual outbreak with regard to its virulence and evolution. Using multilocus sequence typing (MLST), we classified the isolated strains into a single sequence type (ST), ST-7 of the ST-1 complex. The ST-1 complex is strongly associated with cases of septicemia and meningitis worldwide (*8*). *S. suis* ST-7 expressed the proposed virulence markers muramidase-released protein (MRP), extracellular protein factor (EF), and hemolysin (named suilysin) (*9*–*11*) and was markedly

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more cytototoxic to human peripheral blood mononuclear cells (PBMC) than a representative ST-1 strain.

Methods

Bacterial Isolation and Identification

All α-hemolytic streptococcal colonies grown on sheep blood agar from specimens obtained from normally sterile sites on humans or diseased pigs were identified as *S. suis* by API-20 STREP system (Biomerieux, WeTech, Beijing, People's Republic of China) (Table). The specific serotype of the isolates was characterized as *S. suis* capsular type 2 by using *S. suis* antisera specific against individual serotypes (provided by the Statens Serum Institut, Copenhagen, Denmark) and confirmed by the coagglutination test, as previously reported (*12*). PCR assays were used to detect the genes coding for 16S rRNA of *S. suis*, for the capsule of *S. suis* serotype 2 (*cps2J*), and for MRP, suilysin (*sly*), and EF, as reported (*9*,*10*,*13*). PCR results were confirmed by sequencing the synthesized fragments. The expression of the virulence markers MRP, suilysin, and EF was confirmed at the protein level by Western blotting bacterial culture supernatants as previously described (*11*).

Pulsed-field Gel Electrophoresis (PFGE) Analysis

The protocol described by Berthelot-Hérault et al., with modification, was used (*14*). Cells were restricted with 25 U of *Sma*I (Promega, Sino-American Biotechnology Co, Beijing, People's Republic of China). DNA fragments were resolved by PFGE with 1% SeaKem Gold agarose gels and the CHEF-DR III system (Bio-Rad, Beijing, People's Republic of China). *Salmonella enterica* serovar Braenderup H9812 restricted with *Xba*I was used for molecular weight and size determinations (*15*). Similarities between restriction endonuclease digestion profiles were analyzed by using BioNumerics software (Applied Maths, Kortrijk, Belgium).

MLST and Phylogenetic Analysis

Seven housekeeping gene loci described by King et al. for MLST analysis of *S. suis* were used in this study: *cpn*60, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS* coding for a 60-kDa chaperonin, a putative peroxide resistance protein, a homologous recombination factor, a 5-enolpyruvylshikimate 3-phosphate synthase, an aspartokinase/homoserine dehydrogenase, a glucose kinase, and a DNA mismatch repair enzyme, respectively (*8*). PCR products were purified by using QIAquick PCR product purification columns (Qiagen, Gene, Beijing, People's Republic of China) and directly sequenced at both ends with an ABI Prism 3700 DNA analyzer system (Perkin Elmer Applied Biosystems, Wellesley, MA, USA) (*8*). For each isolate, the alleles at each of the 7 loci defined the allelic profile or ST. MLST information in the *S. sui*s database identified the phylogenetic position of isolates collected in this outbreak investigation (*8*). eBURST was used to identify clonal complexes within *S. suis* and to display the overall structure of the population in the MLST database at http://ssuis.mlst.net (*16*,*17*). This database contains 92 previously described STs derived from isolates from various sources, including diseased pigs and human patients from different countries. Bayesian maximum likelihood trees were reconstructed with MrBayes version 3.1.1, and maximum likelihood trees were made with the HKY85 model (*18*).

Cytotoxicity Assays

Bacterial cytotoxicity to PBMCs from healthy donors was determined as previously described (*19*). PBMCs were seeded at a final concentration of 10^6 cells/mL in 24-well plates and incubated for 4 h at 37°C in the presence of ≈10^7 CFU/mL *S. suis* strain SC84 and SC22 (selected as 2 representative strains from the Sichuan outbreak and isolated from 2 human patients with STSS) or *S. suis* reference strain 31533 used as a control. Strain 31533, isolated from a pig with meningitis in France, was virulent in pig models of infection and toxic to several types of host cells (*19*,*20*). Bacterial cytotoxicity was evaluated by lactate dehydrogenase measurement with Cyto-Tox 96 Cytoxicity Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Percentage of cytotoxicity was calculated as (sample optical density $[OD]_{490}$ – $OD_{0\%}$)/($OD_{100\%}$ – $OD_{0\%}$)×100, where $OD_{0\%}$ represents the OD_{490} of noninfected cells and $OD_{100\%}$ represents the OD_{490} of cells treated with lysis buffer as indicated by the

Table. *Streptococcus suis* capsular type 2 isolates included in this study*

No. isolates tested	Origin (province)	Host	PFGE profile	MLST profile	Comments
84	Sichuan	Human	Type I	ST-7	Summer 2005, outbreak
1	Jiangxi	Human	Type I	ST-7	Summer 2005, sporadic
1	Guangdong	Human	Type I	ST-7	Summer 2005, sporadic
1	Guangxi	Human	Type I	ST-1	Summer 2005, sporadic
1	Guizhou	Human	Type II	ST-1	Summer 2005, sporadic
1	Jiangsu	Human	Type I	ST-7	Summer 1998, outbreak
8	Sichuan	Pig	Type I	ST-7	Summer 2005, outbreak, diseased pigs
3	Jiangxi	Pig	Type I	ST-7	Summer 2005, sporadic, infected pork
1	Jiangsu	Pig	Type I	ST-7	Summer 1998, outbreak, diseased pig

*PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing.

manufacturer. Empty wells with cell culture medium alone served as blanks.

Results

In total, 99 *S. suis* strains were isolated in this study (Table): 88 from human patients and 11 from diseased pigs. Of the 88 human isolates, 1 each was from Guangdong, Jiangxi, Guizhou, and Guangxi. From the Sichuan outbreak in the summer of 2005, we found 84 human isolates, distributed in 63 administrative villages of 29 counties, and included them in this study. Two strains, 1 from a human patient and 1 from a diseased pig in Jiangsu during the 1998 *S. suis* outbreak, were also included to determine whether the causative agents for the 2 outbreaks were clonal (*21*).

Of the 11 pig isolates obtained in 2005, 8 were from Sichuan and 3 were from Jiangxi. One of the 8 isolates from Sichuan, named SC16, originated from 1 diseased pig in a herd. The human patient, who slaughtered and processed the first diseased pig in the same herd, became ill 6 hours later and died 18 hours after the onset of illness. The 3 isolates from Jiangxi were isolated from infected meat samples from a cold storage house. The human patient who processed the meat was infected.

All strains were determined as serotype 2 by using diagnostic antiserum against *S. suis* and confirmed by the coagglutination method with specific type 2 antiserum (*12*). The identity of the isolates as *S. suis* serotype 2 was further confirmed by positive PCR for the genes coding for the 16S rRNA of *S. suis* and for the capsule of *S. suis* serotype 2 (*cps2J*).

PCR showed all isolates to be positive for the virulence genes coding for MRP, suilysin (*sly*), and EF (*9*,*10*). PCR results were confirmed with nucleotide sequencing. Ten selected strains tested positive for suilysin, EF, and MRP by Western blot with specific antibodies, as described previously, which confirmed the expression of these virulence-related genes (*11*). We saw no indication that *S. suis* was transmitted between humans, and almost all patients had contact with pigs or infected meat (*22*).

PFGE effectively detected relationships between genetic background, virulence traits, and epidemiologic implications of many bacterial pathogens. Vela et al. analyzed 302 *S. suis* clinical isolates of various serotypes from various countries and identified 60 different pulse types, of which 50% corresponded to a single pulse type. One pulse type represented 46.3% of the swine isolates that may be related to a higher pathogenic potential or to a wider environmental distribution (*23*). In this study, chromosomal DNA digested with *Sma*I was analyzed in the 101 isolates described in the Table. Results showed 2 pulse types. Pulse type I was found in 100 of the 101 isolates, including those from human patients and diseased pigs from Sichuan and 4 other provinces: Jiangxi, Guangdong, Guangxi, and Jiangsu (Figure 1). This finding indicates that pulse type I was the primary cause of this human outbreak and that the causative agent was clonal. Only 1 isolate, GZ1, isolated in August 2005 and having a slightly different PFGE profile, was from a human patient in Guizhou Province (Figure 1).

Although the PFGE-based network, PulseNet International, is used worldwide for molecular epidemiology, no database is available for *S. suis* to enable comparison of genetic relatedness among isolates from various origins. MLST defines strains by using sequences at 7 housekeeping loci and has become the method of choice for addressing questions related to epidemiology, population, and evolutionary biology. The MLST network, http://www.mlst.net, allows laboratories to quickly characterize their strains, relate them to those submitted by others, and compare them with the pathogen populations as a whole through the Internet (*16*,*24*). The selected 7 housekeeping gene loci from all 101 *S. suis* isolates were amplified and sequenced. DNA sequences from the 7 loci of all isolates were determined (DQ205243-51), and allelic profiles were assigned and submitted to the MLST database for *S. suis* (http://ssuis.mlst.net) (*8*). In our study, 99 of 101 isolates were identified as ST-7, including all 92 isolates from human patients and diseased pigs collected during the Sichuan outbreak in 2005, as well as the 2 isolates from the Jiangsu outbreak in 1998 (1 from a human patient and 1 from a diseased pig). In addition, the isolates from Jiangxi (1 human and 3 pig isolates) and Guangdong

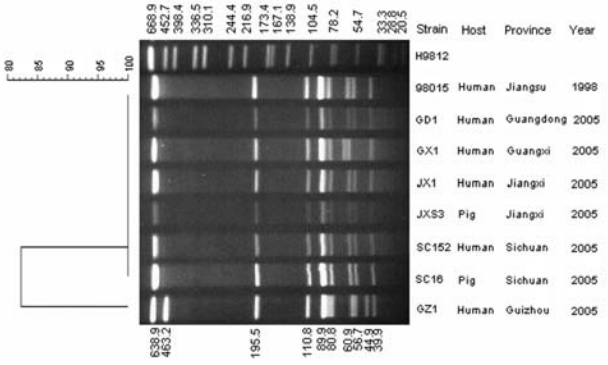

Figure 1. Pulsed-field gel electrophoresis (PFGE) profiles of selected isolates from various parts of China. All isolates of *Streptococcus suis* were digested with *Sma*I. The molecular size of each restriction fragment was calculated with the Comparative Quantification/Polymorphism analysis feature of the Molecular Analyst Fingerprinting Plus software (Quantity One version 4.0, Bio-Rad, Beijing, People's Republic of China) based on mobility of *S. suis* isolates on the same gel with *Salmonella enterica* serovar Braenderup H9812 digested with *Xba*I, as universal standard of PulseNet International. Clustering of PFGE patterns was performed by an unweighted paired group with arithmetic averaging (UPGMA). The dendrogram of PFGE patterns of isolates tested were drawn with PulseNet software BioNumerics, with a 1.5% position tolerance and 1% optimization.

(1 human isolate) obtained during summer 2005 were also assigned to ST-7. One human isolate from a patient in Guizhou (strain GZ1) and 1 human isolate from Guangxi (strain GX1) were identified as ST1 (DQ205251). Strain GZ1 showed a PFGE profile that differed slightly from that of the Sichuan isolates (Figure 1, pulse type II). However, strain GX1 had a pulse type I profile similar to that of the Sichuan isolates. These results indicate that MLST is a more powerful discriminatory and epidemiologic tool.

To gain information on the origin and relationships between ST-7 and other STs, we evaluated the population genetic diversity of the entire *S. suis* MSLT database with eBURST, which divides an MLST dataset of any size into groups of related isolates and clonal complexes, predicts the ancestral genotype for each clonal complex, and computes the bootstrap relatedness value. Using this technique, we showed bacterial clonal diversity in *S. suis* and provided evidence for the emergence of new clones of particular clinical relevance (*17*). The population snapshot of all isolates of *S. suis* generated by eBURST, including our 101 isolates along with all 294 isolates with available MLST data, showed 6 major clusters of related STs and numerous unlinked STs. Of unlinked STs, we noted 6 ST complexes with single-locus variants and some with double-locus variants (*8*,*17*). Interspersed among these clonal complexes were minor groups, typically joined doublets and individually unlinked STs that were not single-locus variants of any other STs in the database (Figure 2).

The 6 major clonal complexes with single- and double-locus variants were ST-1, ST-17, ST-27, ST-61, ST-29, and ST-87 (Figure 2). The major clonal complex of *S. suis*, ST-1, contained isolates from humans and pigs that had invasive disease or were healthy carriers and included, besides serotype 2, isolates of serotypes 1, 1/2, 3, 8, 14, and 1/14. ST-1, original strain of the ST-1 complex (with 100% bootstrap support), includes pig isolates from England (83), Spain (44), the Netherlands (3), France (4), and Hong Kong (5); it is the predominant ST responsible for most swine infections worldwide.

ST-7 may be derived from ST-1, the primary strain of the clonal ST-1 complex, and it may have caused the human infections in Sichuan Province. ST-7 (ST profile 1,1,1,1,1,1,3) (DQ205243-50) and ST-1 (ST profile 1,1,1,1,1,1,1) (DQ205243-51) may have diversified by a stepwise accumulation of point mutations. Furthermore, ST-7 may have only very recently diverged, since it showed a high level of similarity in terms of both allelic profiles and sequences to their nonidentical alleles. Thus, the closely related ST-1 and ST-7 share 6 identical loci and have 1 locus, *thyA*, differing only at a single nucleotide (Figure 2). Because the housekeeping genes are recognized as the stable core of the bacterial genome, this divergence further shows the emergence of a new virulent ST.

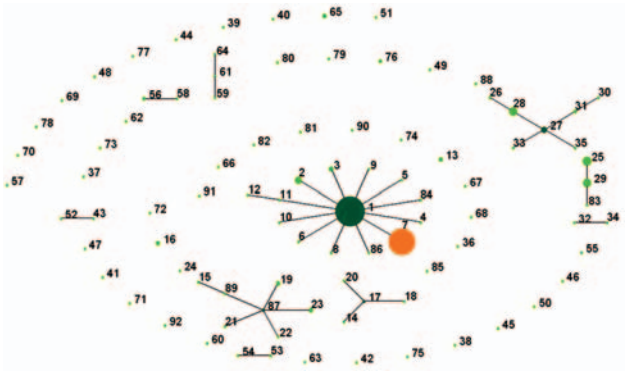

Figure 2. Population snapshot of *Streptococcus suis*. The entire *S. suis* multilocus sequence type database is displayed as a single eBURST diagram. The 6 major sequence type (ST) complexes are each denoted by a number. The patterns of descent within these ST complexes are discussed in the text. Primary founders (dark green) are positioned centrally in the cluster, and subgroup founders are shown in light green, except ST-7, which is shown in orange to emphasize its importance. The area of each circle in the diagram corresponds to the abundance of the isolates of the ST in the input data.

Using eBURST analysis of the MSLT database, we also examined isolates of *S. suis* serotype 2 from human patients or diseased pigs that have been shown experimentally to be highly virulent in porcine models of infection (*8*,*20*,*25*). Six of the 7 so-called highly virulent strains belonged to ST-1, including 1 strain isolated from a patient with meningitis in England (H11/1) and 1 strain isolated from an abattoir worker with meningitis in Hong Kong (87555). Another highly virulent strain (89-1591) isolated from a pig with septicemia was ST-25, a single-locus variant of the ST-29 complex (*26*).

Discussion

Strains isolated from human patients (including those from the MLST database and this study) can be regrouped in either ST-1, ST-6, ST-7, and ST-84 (ST-1 complex) or ST-25 (ST-29 complex) by MLST. ST-1 includes 10 isolates of *S. suis* serotype 2 recovered from human infections in France (n = 4), England (n = 1), and Hong Kong. The Hong Kong strains were isolated in 1985 (n = 1), 1995 (n = 3), and 1997 (n = 1). In addition, ST-1 also includes 1 strain of *S. suis* serotype 14 isolated from a patient in England. ST-25 contains the only 3 serotype 2 strains isolated from human patients in Canada (*27*,*28*). ST-6 contains a strain of serotype 14 isolated from a human patient in the Netherlands (*29*). Before our study, only 1 member of ST-7 had been isolated from the blood of a patient with sepsis in Hong Kong in 1996 (*8*,*30*). The ST-1 and ST-29

complexes are highly virulent clones of *S. suis* populations, and the ST-1 clonal complex is the major complex of *S. suis* with public health ramifications and is responsible for most human infections. The 3 isolates in ST-25 have none of the recognized virulence genes in *S. suis*: *ef, mrp,* and *sly*. Therefore, we propose that the ST-1 clonal complex is the dominant highly virulent complex and has the potential to cause large human *S. suis* infection outbreaks. The potential of ST-7 as an emerging virulent clone within the ST-1 complex was further suggested by results obtained by using the cytotoxicity assay with human PBMCs. As shown in Figure 3, live bacterial cell suspensions prepared from *S. suis* strains SC84 and SC22 (ST-7), isolated from human patients with STSS in Sichuan, were significantly more toxic to PBMCs than the *S. suis* reference strain 31533 (ST-1) when cells were incubated >2 hours under similar conditions of bacterial growth. Strain 31533 was virulent in mouse and pig models of infection and was toxic to several types of host cells, as demonstrated by in vitro assays (*19*). Thus, this difference in cytotoxicity is not only significant but also relevant to the fulminant characteristics of the Sichuan outbreak.

This hypothesis is further supported by phylogenetic analysis of the sequences from 92 STs of the 7 *S. suis* housekeeping genes (Figure 4). To show the phylogenetic relationships between the ST-1 complex and genetic populations of *S. suis*, we examined the possibility that phylogenetic data were sufficient to construct a relatedness tree among members of STs and, in particular, for the virulent clonal ST-1 complex. Phylogenetic analysis showed 6 lineages within the *S. suis* population. The previously identified complexes ST-61, ST-27, and ST-87 were placed in lineages 2, 5, and 6, respectively; most of the STs have only a single isolate. The ST-29 complex that contains human isolates of ST-25 is in lineage 5. The clonal ST-1 complex forms a single lineage and appears to be dominant in various locations (*8*). It has 4 STs for human isolates, ST-1, ST-6, ST-7, and ST-84, which may have evolved from a common ancestor (Figure 4). In this complex, *S. suis* ST-7 emerged first in Hong Kong in 1996, caused 28 cases in Jiangsu Province in 1998, and was responsible for the largest outbreak of human *S. suis* infection in history that occurred in Sichuan Province, China, in 2005 and resulted in 215 cases with 38 deaths (*8*,*21*,*30*).

The high death rate in the Sichuan outbreak is of great concern since few human *S. suis* infections are fatal. This increased virulence may be related to a horizontal gene transfer of a possible new toxin or superantigen consistent with the most relevant clinical manifestation of this outbreak, i.e., STSS. Recently, resistance gene exchanges were reported between porcine *S. suis* and various human streptococcal species, including *S. pyogenes*. Thus, gene transfer

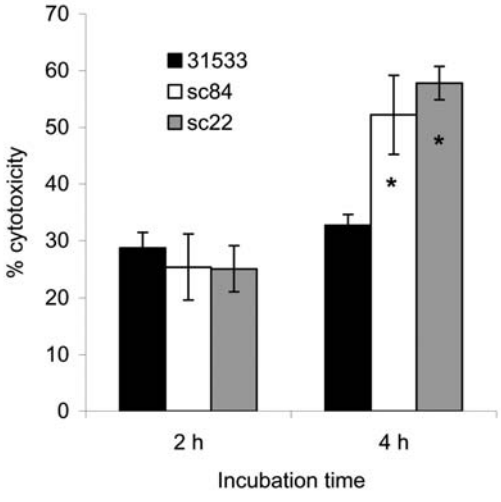

Figure 3. Cytotoxicity of *Streptococcus suis* sequence type (ST) 7 to human peripheral blood mononuclear cells. Data were collected from 3 experiments, which were performed in triplicate and expressed as the mean percentage of cytotoxicity, plus or minus standard deviations. *p<0.001 (compared to the value of *S. suis* ST-1 representative strain 31533) as determined by analysis of variance with SAS version 8 software (SAS Institute, Cary, NC, USA).

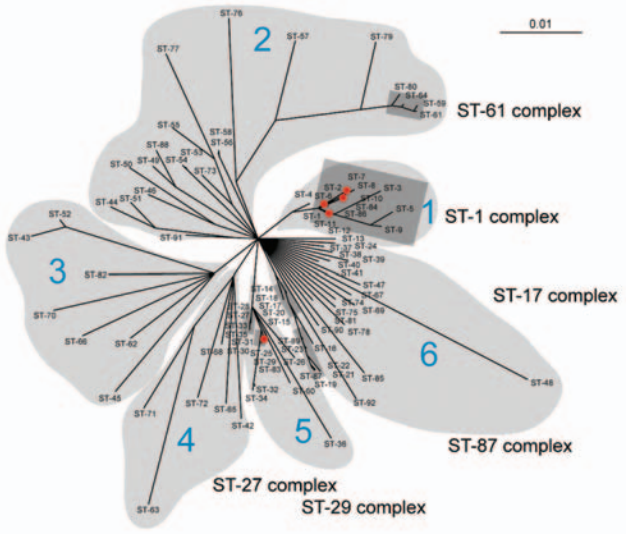

Figure 4. Unrooted Bayesian tree of the concatenated sequence of the 92 sequence types (STs) of *Streptococcus suis*. The tree was constructed by using MrBayes (version 3.1.1) according to the HKY85 model of DNA substitution with no rate variation across sites. Four Markov chains were run for a million generations, and the Markov chain is sampled every 100 generations (*18*). The sampled parameter values were summarized by discarding the first 2,000 samples as burn-in. On the basis of the last 9,000 samples taken from the posterior probability distribution, a 50% majority rule consensus tree was computed. The posterior probability given on each branch is a percentage of these trees supporting each node. The 6 lineages defined in this study are shadowed in light gray. The 6 major clonal complexes identified previously by King et al. (*8*) as well as in this study are shadowed in dark gray. The five STs containing isolates from human invasive disease are shown as red dots. A larger version of this figure is available online at http://www.cdc.gov/ncidod/EID/vol12no08/06-0232-G4.htm

from porcine to human streptococci and vice versa is possible, albeit at a low frequency (*31*). Therefore, *S. suis* ST-7 might be an emerging virulent infectious disease. This unusual *S. suis* clone may spread further across China and to the rest of the world; spread may become more pronounced with the rapid development of the export business.

Acknowledgments

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Dr Ye is a microbiologist. Her main research interest is in emerging infectious diseases. She is a group leader for the study of *S. suis* infection in China.

References

1. Baddeley PG. *Streptococcus suis* infection. Occup Med (Lond). 1995;45:222.
2. Halaby T, Hoitsma E, Hupperts R, Spanjaard L, Luirink M, Jacobs J. *Streptococcus suis* meningitis, a poacher's risk. Eur J Clin Microbiol Infect Dis. 2000;19:943–5.
3. Juncal AR, Pardo F, Rodriguez I, Perez del Molino ML. Meningitis by *Streptococcus suis* [article in Spanish]. Enferm Infecc Microbiol Clin. 1997;15:120–1.
4. Staats JJ, Feder I, Okwumabua O, Chengappa MM. *Streptococcus suis*: past and present. Vet Res Commun. 1997;21:381–407.
5. Dupas D, Vignon M, Geraut C. *Streptococcus suis* meningitis. A severe noncompensated occupational disease. J Occup Med. 1992;34:1102–5.
6. Gottschalk M, Segura M. The pathogenesis of the meningitis caused by *Streptococcus suis*: the unresolved questions. Vet Microbiol. 2000;76:259–72.
7. Suankratay C, Intalapaporn P, Nunthapisud P, Arunyingmongkol K, Wilde H. *Streptococcus suis* meningitis in Thailand. Southeast Asian J Trop Med Public Health. 2004;35:868–76.
8. King SJ, Leigh JA, Heath PJ, Luque I, Tarradas C, Dowson CG, et al. Development of a multilocus sequence typing scheme for the pig pathogen *Streptococcus suis*: identification of virulent clones and potential capsular serotype exchange. J Clin Microbiol. 2002;40: 3671–80.
9. Wisselink HJ, Reek FH, Vecht U, Stockhofe-Zurwieden N, Smits MA, Smith HE. Detection of virulent strains of *Streptococcus suis* type 2 and highly virulent strains of *Streptococcus suis* type 1 in tonsillar specimens of pigs by PCR. Vet Microbiol. 1999;67:143–57.
10. Staats JJ, Plattner BL, Stewart GC, Changappa MM. Presence of the *Streptococcus suis* suilysin gene and expression of MRP and EF correlates with high virulence in *Streptococcus suis* type 2 isolates. Vet Microbiol. 1999;70:201–11.
11. Berthelot-Herault F, Morvan H, Keribin AM, Gottschalk M, Kobisch M. Production of muraminidase-released protein (MRP), extracellular factor (EF) and suilysin by field isolates of *Streptococcus suis* capsular types 2, 1/2, 9, 7 and 3 isolated from swine in France. Vet Res. 2000;31:473–9.
12. Higgins R, Gottschalk M. An update on *Streptococcus suis* identification. J Vet Diagn Invest. 1990;2:249–52.
13. Marois C, Bougeard S, Gottschalk M, Kobisch M. Multiplex PCR assay for detection of *Streptococcus suis* species and serotypes 2 and 1/2 in tonsils of live and dead pigs. J Clin Microbiol. 2004;42:3169–75.
14. Berthelot-Herault F, Marois C, Gottschalk M, Kobisch M. Genetic diversity of *Streptococcus suis* strains isolated from pigs and humans as revealed by pulsed-field gel electrophoresis. J Clin Microbiol. 2002;40:615–9.
15. Hunter SB, Vauterin P, Lambert-Fair MA, van Duyne MS, Kubota K, Graves L, et al. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. J Clin Microbiol. 2005;43:1045–50.
16. Aanensen DM, Spratt BG. The multilocus sequence typing network: mlst.net. Nucleic Acids Res. 2005;33:W728–33.
17. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186:1518–30.
18. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572–4.
19. Vanier G, Segura M, Friedl P, Lacouture S, Gottschalk M. Invasion of porcine brain microvascular endothelial cells by *Streptococcus suis* serotype 2. Infect Immun. 2004;72:1441–9.
20. Vecht U, Wisselink HJ, Stockhofe-Zurwieden N, Smith HE. Characterization of virulence of the *Streptococcus suis* serotype 2 reference strain Henrichsen S 735 in newborn gnotobiotic pigs. Vet Microbiol. 1996;51:125–36.
21. Zhu F, Yang H, Hu X, Wang H, Wang G, Song Y, et al. Homogeneity study on the *Streptococcus suis* isolated from human and swine [article in Chinese]. Zhonghua Liu Xing Bing Xue Za Zhi. 2000;21:427–9.
22. Yu H, Jing H, Chen Z, Zheng H, Zhu X, Wang H, et al. Human *Streptococcus suis* outbreak, Sichuan, China. Emerg Infect Dis. 2006;12:914–20.
23. Vela AI, Goyache J, Tarradas C, Luque I, Mateos A, Moreno MA, et al. Analysis of genetic diversity of *Streptococcus suis* clinical isolates from pigs in Spain by pulsed-field gel electrophoresis. J Clin Microbiol. 2003;41:2498–502.
24. Cooper JE, Feil EJ. Multilocus sequence typing–what is resolved? Trends Microbiol. 2004;12:373–7.
25. Galina L, Pijoan C, Sitjar M, Christianson WT, Rossow K, Collins JE. Interaction between *Streptococcus suis* serotype 2 and porcine reproductive and respiratory syndrome virus in specific pathogen-free piglets. Vet Rec. 1994;134:60–4.
26. Quessy S, Dubreuil JD, Caya M, Higgins R. Discrimination of virulent and avirulent *Streptococcus suis* capsular type 2 isolates from different geographical origins. Infect Immun. 1995;63:1975–9.
27. Michaud S. *Streptococcus suis* meningitis: first case reported in Quebec. Can J Infect Dis. 1996;7:329–31.
28. Trottier S, Higgins R, Brochu G, Gottschalk M. A case of human endocarditis due to *Streptococcus suis* in North America. Rev Infect Dis. 1991;13:1251–2.
29. Gottschalk M, Higgins R, Jacques M, Mittal KR, Henrichsen J. Description of 14 new capsular types of *Streptococcus suis*. J Clin Microbiol. 1989;27:2633–6.
30. Kay R, Cheng AF, Tse CY. *Streptococcus suis* infection in Hong Kong. QJM. 1995;88:39–47.
31. Martel A, Decostere A, Leener ED, Marien M, Graef ED, Heyndrickx M, et al. Comparison and transferability of the erm (B) genes between human and farm animal streptococci 6. Microb Drug Resist. 2005;11:295–302.

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Carbapenem Resistance in *Klebsiella pneumoniae* Not Detected by Automated Susceptibility Testing

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Detecting β-lactamase–mediated carbapenem resistance among *Klebsiella pneumoniae* isolates and other *Enterobacteriaceae* is an emerging problem. In this study, 15 bla_{KPC}-positive *Klebsiella pneumoniae* that showed discrepant results for imipenem and meropenem from 4 New York City hospitals were characterized by isoelectric focusing; broth microdilution (BMD); disk diffusion (DD); and MicroScan, Phoenix, Sensititre, VITEK, and VITEK 2 automated systems. All 15 isolates were either intermediate or resistant to imipenem and meropenem by BMD; 1 was susceptible to imipenem by DD. MicroScan and Phoenix reported 1 (6.7%) and 2 (13.3%) isolates, respectively, as imipenem susceptible. VITEK and VITEK 2 reported 10 (67%) and 5 (33%) isolates, respectively, as imipenem susceptible. By Sensititre, 13 (87%) isolates were susceptible to imipenem, and 12 (80%) were susceptible to meropenem. The VITEK 2 Advanced Expert System changed 2 imipenem MIC results from ≥16 μg/mL to ≤2 μg/mL but kept the interpretation as resistant. The recognition of carbapenem-resistant *K. pneumoniae* continues to challenge automated susceptibility systems.

Carbapenem resistance among the *Enterobacteriaceae* is emerging in the United States, particularly on the East Coast (*1*–*6*). Resistance to the most widely used carbapenems, i.e., imipenem and meropenem, can be mediated by a variety of mechanisms, including β-lactamases, porin changes, and changes in penicillin-binding proteins (*1*,*7*,*8*). KPC enzymes are among the most common β-lactamases mediating carbapenem resistance among isolates of *Enterobacteriaceae* (*1*–*6*). KPC enzymes are class A β-lactamases that mediate resistance to extended-spectrum cephalosporins in addition to carbapenems; these β-lactamases are usually plasmid encoded.

Clinical microbiology laboratories have often found it difficult to achieve accurate susceptibility testing results for carbapenem drugs. Early studies documented false resistance to imipenem due to degradation of the drug (*9*); later studies with the VITEK system (bioMérieux, Durham, NC, USA) demonstrated false resistance, specifically with *Proteus mirabilis* (*10*). Several recent proficiency testing studies have shown problems of both false resistance and false susceptibility with imipenem and meropenem among a variety of enteric species (*11*,*12*). Even quality control measures fail to detect all false resistance problems (*13*).

Yigit and colleagues described the KPC-1 β-lactamase in 2001 (*1*). The β-lactamase was identified in an imipenem-resistant isolate of *Klebsiella pneumoniae* from the United States. Subsequently, 3 additional KPC-type β-lactamases have been described from *Salmonella*, *K. oxytoca*, *Enterobacter cloacae*, and other *K. pneumoniae*; these differ in amino acid sequence from each other, typically by 1 or 2 amino acids (*2*–*6*). Bratu and colleagues reported false-susceptible results for *K. pneumoniae* isolates with the MicroScan WalkAway system (Dade MicroScan, Inc., West Sacramento, CA, USA), which were attributed in part to low inoculum size (*14*). Similar problems with false-susceptible results were noted with the VITEK system (*15*). The goal of this study was to conduct a rapid assessment of currently available antimicrobial susceptibility testing methods to determine whether these methods were capable of consistently detecting KPC-mediated carbapenem resistance in fresh clinical isolates of *K. pneumoniae.*

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Materials and Methods

Bacterial Isolates

To achieve a diversity of β-lactam resistance phenotypes, we selected 15 isolates of *K. pneumoniae* from 4 hospitals in New York City, all serviced by a central microbiology laboratory, on the basis of varying susceptibility patterns to imipenem, meropenem, and extended-spectrum cephalosporins. The isolates were from a variety of body sites (blood, sputum, and urine) and obtained in a 1-month period in 2005. The carbapenem-resistant quality control isolates *K. pneumoniae* 1534 (containing the KPC-1 carbapenemase) and *Serratia marcescens* 525 (which contains an SME-like β-lactamase) were from the Project ICARE (Intensive Care Antimicrobial Resistance Epidemiology) strain collection (*1*,*11*). The imipenem MICs for the isolates and the study identification of their hospital of origin are shown in Table 1.

Reference Susceptibility Testing Methods

The antimicrobial susceptibility profiles of the isolates were determined by the broth microdilution method with cation-adjusted Mueller-Hinton broth (BD Diagnostic Systems [BDDS], Sparks, MD, USA), as described in the National Committee for Clinical Laboratory Standards (NCCLS, Wayne, PA, USA) (now known as the Clinical and Laboratory Standards Institute [CLSI]) publication M7-A6 (*16*). Disk diffusion was performed as described in NCCLS document M2-A8 (*17*). Interpretations of MIC and disk diffusion results were made by using CLSI document M100-S15 (*18*).

Commercial Susceptibility Testing and Molecular Methods

The Etest method (AB Biodisk, Solna, Sweden) was performed as described by the manufacturer with Mueller-Hinton agar (BDDS); tests were interpreted at 18–20 h. The MicroScan WalkAway (Dade MicroScan, Inc.), BD Phoenix (BDDS), Sensititre AutoReader (Westlake, OH, USA), VITEK Legacy (bioMérieux), and VITEK 2 (bioMérieux) systems were tested according to manufacturers' protocols. The panels and cards used are listed in Table 2. All systems were tested with inocula from the same subculture. All strains for which the commercial MIC results were discrepant with MIC results from the broth microdilution reference method were retested by using all methods.

The carbapenem inactivation assay was performed on Mueller-Hinton agar with imipenem and meropenem disks as described by Yigit et al. (*1*). Isoelectric focusing was performed on crude lysates of isolates as previously described (*1*,*2*).

For detection of bla_{KPC} genes, a 489-bp internal gene fragment was amplified by using forward (5′-CTTGCTGCCGCTGTGCTG-3′) and reverse (5′-GCAGGTTCCGGTTTTGTCTC-3′) oligonucleotide primers where the 5′ base of each primer corresponds to position 223 or 711, respectively, with regard to the translational start site (bla_{KPC-1} numbering, GenBank accession no. AF297554). PCR reagents included a final concentration of 0.5 μmol/L of each primer and 2 mmol/L $MgCl_2$. An annealing temperature of 60°C was used for amplification.

Table 1. Carbapenem susceptibility and strain typing results for isolates tested in the study*

Organism	Imipenem broth microdilution MIC† (μg/mL) and CLSI interpretation	Meropenem broth microdilution MIC (μg/mL) and CLSI interpretation	PFGE profile	Hospital identification no.
1 *Klebsiella pneumoniae*	8 I	16 R	A	1
2 *K. pneumoniae*	16 R	>16 R	A	1
3 *K. pneumoniae*	16 R	16 R	A	2
4 *K. pneumoniae*	8 I	8 I	A	2
5 *K. pneumoniae*	16 R	>16 R	A	2
6 *K. pneumoniae*	16 R	16 R	A	2
7 *K. pneumoniae*	16 R	16 R	A	3
8 *K. pneumoniae*	32 R	16 R	A	4
9 *K. pneumoniae*	16 R	>16 R	B	1
10 *K. pneumoniae*	16 R	>16 R	B	1
11 *K. pneumoniae*	16 R	16 R	C	4
12 *K. pneumoniae*	16 R	16 R	D	1
13 *K. pneumoniae*	16 R	16 R	E	4
14 *K. pneumoniae*	16 R	>16 R	F	1
15 *K. pneumoniae*	16 R	>16 R	F1	4
K. pneumoniae 1534 (control)	16 R	>16 R	NA	NA
Serratia marcescens 525 (control)	>16 R	>16 R	NA	NA

*CLSI, Clinical and Laboratory Standards Institute; PFGE, pulsed-field gel electrophoresis; NA, not applicable.
†Interpretations of MIC results used CLSI criteria (*18*); I, intermediate; R, resistant.

Table 2. Summary of antimicrobial susceptibility testing results for 15 test isolates*

Method (software)	Card/panel	Imipenem results (n = 15)			Meropenem results (n = 15)		
		Resistant	Intermediate	Susceptible	Resistant	Intermediate	Susceptible
Broth microdilution	In-house frozen panel	13	2	0	14	1	0
Disk diffusion	BDDS disks	3	11	1	10	5	0
MicroScan (LabPro1.51, Alert 1.50)	Neg combo 32	7	7	1	13	1	1
Phoenix (4.05W/3.81A)	NMIC/ ID-104	5	8	2	12	1	2
Sensititre AutoReader (3.0.8 SP2)	GN2F	0	2	13	0	3	12
VITEK (R10.01)	Superflex GNS 122 and 127	5	0	10	2	3	10
VITEK 2* (R04.01)	GN07	4	6	5	4	4	5

*No meropenem interpretations were given by the Advanced Expert System for 2 organisms.

For DNA sequence determination, a 989-bp PCR product that included the entire bla_{KPC} structural gene was amplified by using oligonucleotide primers as previously described (*4*). Products were purified on QIAquick spin columns (Qiagen, Chatsworth, CA, USA). The nucleotide sequences of both strands of the bla_{KPC} gene from isolates 4 and 11 were determined from independent amplification products by using previously described primers (*1*). Cycle sequencing reactions were performed in a GeneAmp PCR system 9700 thermal cycler with the ABI BigDye Terminator v3.1 cycle sequencing kit (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA). Products from sequencing reactions were purified on Cetri-Sep spin columns (Princeton Separations, Adelphia, NJ, USA) before analysis on an ABI 3130xl Genetic Analyzer. DNA sequencing data were analyzed by using DNASIS for Windows (Hitachi Software Genetic Systems, San Francisco, CA, USA).

Results

Carbapenem Testing with VITEK Legacy

Initial imipenem and meropenem susceptibility test results for 15 isolates of *K. pneumoniae* tested with the VITEK Legacy system with GNS 122 and 127 panels (flex system) in 1 hospital laboratory in New York City in a 1-month period in 2005 yielded a range of imipenem and meropenem MICs from susceptible (MIC ≤4 μg/mL) to resistant (MIC ≥16 μg/mL) (data not shown). One isolate was imipenem resistant (MIC >16 μg/mL) but meropenem susceptible (≤4 μg/mL) on day 1, but on retesting the following day was meropenem resistant (16 μg/mL) and imipenem susceptible (≤4 μg/mL). Imipenem Etest results, which were set up to arbitrate the conflicting results, were difficult to interpret because of variable numbers of colonies within the ellipses of inhibition (Figure). A carbapenem inactivation assay performed on 1 isolate indicated that it contained a carbapenem-inactivating enzyme (data not shown). The 15 isolates, which were collected from 4 different hospitals in New York City, were sent to the Centers for Disease Control and Prevention (Atlanta, GA, USA) for additional testing.

Characterization of bla_{KPC}-containing *K. pneumoniae* Strains

By using the broth microdilution reference method, all the isolates were either intermediate or resistant to both imipenem and meropenem (Table 2). Two known imipenem-resistant isolates, *K. pneumoniae* 1534 and *S. marcescens* 525, were included as controls. Eight of 15 isolates from the 4 hospitals had the same pulsed-field gel electrophoresis (PFGE) type (data not shown), although their antibiograms varied for several antimicrobial agents (data not shown). Two isolates had a similar pattern but because of a 3-band difference were designated as type B. The remaining isolates showed patterns unrelated to types A or B (Table 1). All 15 isolates demonstrated 5 β-lactamase bands by isoelectric focusing (pIs = 5.4, 6.8, 7.0, 8.1, and 8.2), 1 of which was consistent with a KPC β-lactamase (pI = 6.8) (*1*,*2*). A 489-bp gene fragment was amplified from all 15 carbapenem-resistant *K. pneumoniae* isolates by using bla_{KPC}-specific oligonucleotide primers. DNA sequence analysis of purified PCR products that included the entire coding region of the bla_{KPC} genes of isolates 4 and 11 (which had unique PFGE profiles) identified the β-lactamase genes as bla_{KPC-2} and bla_{KPC-3}, respectively.

The 15 isolates were tested for imipenem and meropenem resistance by disk diffusion, Etest, MicroScan WalkAway, BD Phoenix, Sensititre AutoReader, VITEK, and VITEK 2 panels and cards. The results of testing are summarized in Table 2. MicroScan WalkAway reported 1 isolate as susceptible to both imipenem and meropenem, whereas the Phoenix system called 2 isolates susceptible to both imipenem and meropenem. VITEK called 7 isolates (representing 3 different PFGE profiles) susceptible to both

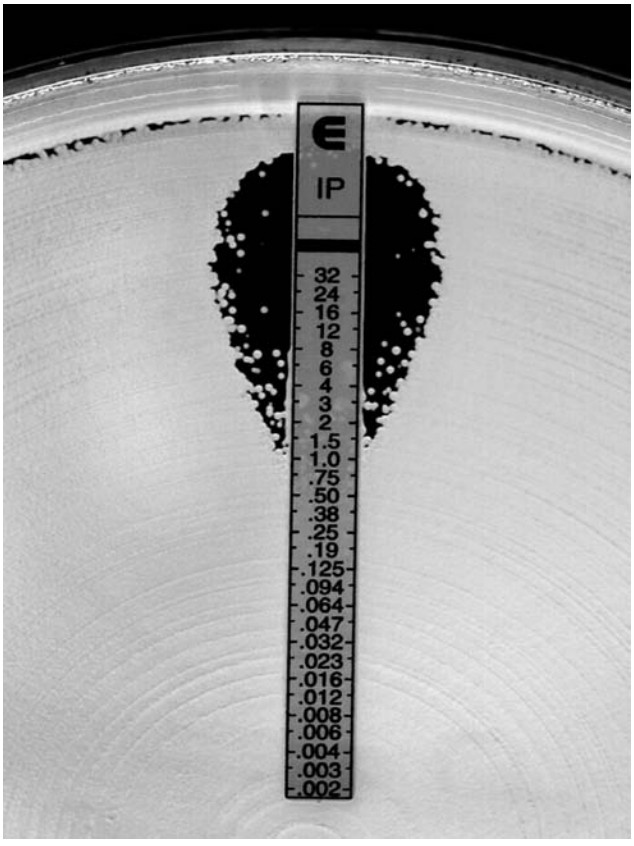

Figure. *Klebsiella pneumoniae* isolate tested with imipenem Etest strip (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar. Inner colonies made determination of the imipenem MIC difficult.

imipenem and meropenem, 3 isolates resistant to imipenem but susceptible to meropenem, 1 isolate susceptible to imipenem but resistant to meropenem, and 2 isolates susceptible to imipenem but intermediate to meropenem. Of the final 2 isolates, 1 was resistant to imipenem and intermediate to meropenem, and the other was resistant to both antimicrobial agents. Thus, 10 (67%) of 15 isolates were interpreted on initial testing as susceptible to imipenem, and 10 were susceptible to meropenem. When the VITEK 2 system was used, 5 (33%) of 15 isolates were reported as susceptible to imipenem. The VITEK 2 Advanced Expert System (AES) used the imipenem results to predict meropenem results; thus, these same 5 isolates were called meropenem susceptible. In addition, as a result of the AES's recognizing unusual susceptibility results in the antibiograms of 2 *K. pneumoniae* isolates, AES did not report an interpretation for meropenem for 2 isolates. Finally, the VITEK 2 reported 2 isolates as imipenem resistant and 1 isolate as imipenem intermediate, although the MICs reported by AES were listed as ≤2 μg/mL. Aside from these, all AES categorical interpretations were in agreement with the original VITEK 2 MIC results.

When Sensititre panels were used, 10 (67%) of 15 isolates were reported as susceptible to both imipenem and meropenem, 2 isolates were reported as imipenem intermediate and meropenem susceptible, and 3 isolates were reported as imipenem susceptible and meropenem intermediate. Thus, on initial testing, 13 (87%) of 15 isolates were reported as imipenem susceptible, and 12 (80%) of 15 were meropenem susceptible. Repeat testing of strain 4 yielded imipenem- and meropenem-resistant results for MicroScan, BD Phoenix, VITEK Legacy, and VITEK 2. However, the Sensititre AutoReader results showed the isolate as susceptible to imipenem and meropenem. Retesting of strain 14 on the BD Phoenix showed the isolate as resistant to imipenem and meropenem. However, with VITEK Legacy, the isolate remained susceptible to imipenem, but the response to meropenem switched from 8 μg/mL (intermediate) to ≤4 μg/mL (susceptible), which confirmed the observations of flip-flopping (i.e., reversing) results from the New York City laboratory. The Sensititre AutoReader results for strain 14 remained susceptible on repeat testing; MicroScan results remained resistant.

Discussion

Detecting KPC-mediated carbapenem resistance in *K. pneumoniae* isolates remains a challenge for many automated susceptibility testing systems. Although we used a total of only 17 isolates in this study (including 1 *S. marcescens* and 1 *K. pneumoniae* control), the isolates and controls represented 4 different carbapenemases (KPC-1, KPC-2, KPC-3, and an SME-like β-lactamase), 7 PFGE types, and variable imipenem and meropenem resistance profiles. Indeed, an important observation of this study is that the carbapenem-resistance profiles of the isolates varied from day to day, sometimes reversing from imipenem resistant/ meropenem susceptible to imipenem susceptible/meropenem resistant**.** Although in our study the MicroScan and BD Phoenix systems produced results that were more consistent with those with the reference testing systems than those with the VITEK and Sensititre AutoReader systems, problems detecting carbapenem resistance were still evident with the former systems. Bratu et al. suggested that part of the variability in detecting imipenem resistance with automated systems was a result of underinoculating the panels (*14,15*). Repeat testing of isolate 4 in our study with careful attention to inoculum appeared to improve results, which suggests that appropriate inoculum size is, indeed, a critical factor for achieving accurate results. The problem of the VITEK 2 AES reporting imipenem-resistant results as ≤2 μg/mL has apparently been corrected in software version R04.02.

Although we included the Etest method in our study, determining resistance and susceptibility for both imipenem and meropenem with Etest was difficult because

colonies were present within the zones of inhibition. Since we could not achieve consensus on the interpretations among several readers who viewed the results, we did not include the Etest data in our analysis. Ertapenem Etest strips and disks were not tested in this study. This lack of consensus on reading Etest method, which is often used as a secondary testing method to confirm questionable results generated by automated methods, raises the question of which, if any, of the methods are reliable enough to be used for confirmation testing of carbapenem nonsusceptibility, particularly in *K. pneumoniae* isolates. Our data suggest that disk diffusion, especially with meropenem disks, may be used to confirm a carbapenem nonsusceptible result in *K. pneumoniae* isolates, which would warrant further testing. Whether this recommendation will hold true for other species of *Enterobacteriaceae* will require further study. Our data also suggest that if the interpretations of MIC or disk diffusion results for imipenem and meropenem for *K. pneumoniae* are discrepant, isolates should be retested with particular attention to using an adequate inoculum size. If treatment failure with carbapenems is observed for isolates of *K. pneumoniae* that were previously reported as susceptible to carbapenems, repeat testing with a nonautomated method, such as disk diffusion, may be warranted.

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References

1. Yigit H, Queenan AM, Anderson GJ, Sanchez-Sanchez AD, Biddle JW, Steward CD, et al. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob Agents Chemother. 2001;45:1151–61.
2. Yigit H, Queenan AM, Anderson GA, Biddle JW, Steward CD, Domenech-Sanchez A, et al. Characterization of a carbapenem-resistant strain of *Klebsiella oxytoca* harboring the KPC-2 carbapenemase. Antimicrob Agents Chemother. 2003;47:3881–9.
3. Bradford PA, Bratu S, Urban C, Visalli M, Mariano N, Landman D, et al. Emergence of carbapenem-resistant *Klebsiella* spp. possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor resistant TEM-30 beta-lactamases in New York City. Clin Infect Dis. 2004;39:55–60.
4. Smith Moland E, Hanson ND, Herrera VL, Black JA, Lockhart TJ, Hossain A, et al. Plasmid-mediated, carbapenem-hydrolysing β-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. J Antimicrob Chemother. 2003;51:711–4.
5. Bratu S, Landman D, Alam M, Tolentino E, Quale J. Detection of KPC carbapenem-hydrolyzing enzymes in *Enterobacter* spp. from Brooklyn, New York. Antimicrob Agents Chemother. 2005;49:776–8.
6. Woodford N, Tierno PM Jr, Young K, Tysall L, Palepou MF, Ward E, et al. Outbreak of *Klebsiella pneumoniae* producing a new carbapenem-hydrolyzing class A beta-lactamase, KPC-3, in a New York Medical Center. Antimicrob Agents Chemother. 2004;48:4793–9.
7. Yigit H, Anderson GJ, Biddle JW, Steward CD, Rasheed JK, Valera LL, et al. Carbapenem resistance in a clinical isolate of *Enterobacter aerogene*s is associated with decreased expression of OmpF and OmpC analogs. Antimicrob Agents Chemother. 2002;46:3817–22.
8. Villar HE, Danel F, Livermore DM. Permeability to carbapenems of *Proteus mirabilis* mutants selected for resistance to imipenem or other beta-lactams. J Antimicrob Chemother. 1997;40:365–70.
9. White RL, Kays MB, Friedrich LV, Brown EW, Koonce JR. Pseudoresistance of *Pseudomonas aeruginosa* resulting from degradation of imipenem in an automated susceptibility testing system with pre-dried panels. J Clin Microbiol. 1991;29:398–400.
10. Doern GV, Brueggemann AB, Perla R, Daly J, Halkias D, Jones RN, et al. Multicenter laboratory evaluation of the bioMérieux VITEK antimicrobial susceptibility testing system with 11 antimicrobial agents versus members of the Family *Enterobacteriaceae* and *Pseudomonas aeruginosa*. J Clin Microbiol. 1997;35:2115–9.
11. Steward CD, Wallace D, Hubert SK, Lawton R, Fridkin SK, Gaynes RP, et al. Ability of laboratories to detect emerging antimicrobial resistance in nosocomial pathogens: a survey of Project ICARE laboratories. Diagn Microbiol Infect Dis. 2000;38:59–67.
12. Steward CD, Mohammed JM, Swenson JM, Stocker SA, Williams PP, Gaynes RP, et al. Antimicrobial susceptibility testing of carbapenems: multicenter validity testing and accuracy levels of five antimicrobial test methods for detecting resistance in *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates. J Clin Microbiol. 2003;41:351–8.
13. Carmeli Y, Eichelberger K, Soja D, Dakos J, Venkataraman L, deGirolami P, et al. Failure of quality control measures to prevent reporting of false resistance to imipenem, resulting in a pseudo-outbreak of imipenem-resistant *Pseudomonas aeruginosa*. J Clin Microbiol. 1998;36:595–7.
14. Bratu S, Mooty M, Nichani S, Landman D, Gullans C, Pettinato B, et al. Emergence of KPC-possessing *Klebsiella pneumoniae* in Brooklyn, New York: epidemiology and recommendations for detection. Antimicrob Agents Chemother. 2005;49:3018–20.
15. Bratu S, Landman D, Haag R, Recco R, Eramo A, Alam M, et al. Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City. Arch Intern Med. 2005;165:1430–5.
16. National Committee for Clinical Laboratory Standards (NCCLS). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard–sixth edition. NCCLS document M7–A6. Wayne (PA): The Committee; 2003.
17. National Committee for Clinical Laboratory Standards (NCCLS). Performance standards for antimicrobial disk susceptibility tests; approved standard–eighth edition. NCCLS document M2–A8. Wayne (PA): The Committee; 2003.
18. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; Fifteenth informational supplement M100–S15. Wayne (PA): The Institute; 2005.

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VEB-1 Extended-Spectrum β-Lactamase–producing *Acinetobacter baumannii*, France[1]

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VEB-1 extended-spectrum β-lactamase–producing *Acinetobacter baumannii* was responsible for an outbreak in hospitals in France. A national alert was triggered in September 2003 when 4 hospitals reported clusters of *A. baumannii* infection with similar susceptibility profiles. Case definitions and laboratory guidelines were disseminated, and prospective surveillance was implemented; strains were sent to a single laboratory for characterization and typing. From April 2003 through June 2004, 53 hospitals reported 290 cases of *A. baumannii* infection or colonization; 275 isolates were bla_{VEB-1}-positive and clonally related. Cases were first reported in 5 districts of northern France, then in 10 other districts in 4 regions. Within a region, interhospital spread was associated with patient transfer. In northern France, investigation and control measures led to a reduction of reported cases after January 2004. The national alert enabled early control of new clusters, demonstrating the usefulness of early warning about antimicrobial drug resistance.

During the past decade, nosocomial outbreaks of *Acinetobacter baumannii* have been described with increasing frequency, occurring mostly in intensive care units, burn units, and surgical wards (*1*,*2*). Epidemic strains of *A. baumannii* are often resistant to several antimicrobial drugs, which reduces treatment effectiveness. Nosocomial transmission is from patient to patient and associated with environmental reservoirs (*2*). Several risk factors for *A. baumannii* infections have been identified and include severity of underlying disease, duration of hospitalization, invasive procedures, and prior broad-spectrum antimicrobial drug therapy (*2–4*). *A. baumannii* can be detected in patients without infection (i.e., colonization) or as the source in patients with severe infections; the case-fatality ratio varies from 17% to 46% for septicemia and can be as high as 70% for pneumonia (*1*).

A. baumannii is not the most common antimicrobial drug–resistant pathogen in hospitalized patients; it accounted for 1.2% of all nosocomial infections in 2001 in France (*5*). However, increasing therapeutic difficulty caused by resistance is a serious concern (*6–9*). A variety of molecular mechanisms for resistance to broad-spectrum β-lactams have been reported in *A. baumannii*, such as mutations of penicillin-binding proteins and alterations of membrane permeability, but the most common mechanism is attributed to the presence of β-lactamases encoded by either chromosomes or plasmids (*2*,*10*,*11*). Several class A, B, and D β-lactamases (*2*,*8*) as well as chromosome-mediated cephalosporinases (*12*) confer various resistance phenotypes. Moreover, extended-spectrum β-lactamase (ESBL)–producing *A. baumannii* strains have also been described: PER-1 in Turkey, Korea, and France (*13–15*); VEB-1 in France (*4*,*16*); and CTX-M-2 recently in Japan (*17*).

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[2]These authors contributed equally to this work.

The bla_{VEB-1} ESBL gene is located in a class 1 integron initially detected in *Enterobacteriaceae* and *Pseudomonas aeruginosa* from Southeast Asia (*18–20*). Subsequently, it has been described in clonally related *A. baumannii* isolates recovered during an outbreak that lasted 9 months (August 2001–April 2002) in the intensive care unit of a hospital in northern France (*4,16*). In these strains, the location of the bla_{VEB-1} gene on the chromosomes and integrons was identified (*4,16*). One year after this outbreak was controlled, nosocomial infections with this *A. baumannii* strain reemerged in the same area and subsequently spread to hospitals located in other districts in France. We describe the nationwide spread of this strain from April 2003 through June 2004.

In early September 2003, an alert was triggered through the national nosocomial infection notification system when, within a month, 4 hospitals in a single district (Nord) reported 5 clusters of *A. baumannii* infections with a similar susceptibility profile; all *A. baumannii* strains were confirmed positive for VEB-1. In October 2003, the National Institute of Public Health (Institut de Veille Sanitaire [InVS]) alerted all hospitals in France of the emergence of this VEB-1–producing *A. baumannii* strain, disseminated case definitions and laboratory guidelines, and implemented a prospective, laboratory-based national surveillance system.

Materials and Methods

Case Definitions

A probable case was defined as follows: isolation of an *A. baumannii* strain showing a multidrug-resistance profile similar to that of the 2001 outbreak strain, susceptible to only imipenem and colistin (strain AYE [*16*]), from a patient hospitalized in France between April 1, 2003, and June 30, 2004; only 1 isolate per patient was retained for the study period. A confirmed case was defined as a case for which VEB-1 ESBL production had been confirmed by the central laboratory, which used phenotypic (detection of the synergy image) and genotypic (PCR amplification of the bla_{VEB-1} gene) methods. Infection or colonization was ascertained by clinicians according to national case definitions for nosocomial infections adapted from the Centers for Disease Control and Prevention (*21*).

Epidemiologic Investigation

Case definitions were dispatched to hospital laboratories and infection control units. Cases had to be reported to regional infection control coordinating centers (CCLIN) (*22*) and local health departments through the national nosocomial infection notification system. In this system, implemented in August 2001, baseline reporting requirements use specific criteria; 1 of them is about rare microorganisms, depending on virulence and antimicrobial drug susceptibility (*23*). No list of microorganisms or resistance phenotypes exists; reports are based on the epidemiologic knowledge of the infection control units. Hospitals report $\geq$1 nosocomial infections on a simple form, which summarizes cases and investigations and allows hospitals to request assistance when needed. For the purpose of this investigation, this system was reinforced as hospitals were asked to report not only *A. baumannii* infections but also instances of colonization and to send bacterial strains to a central laboratory. All reported cases were investigated by infection control units, local health departments, and CCLIN, the latter offering on-site assistance to hospitals when needed. Data on all cases were centralized and analyzed by InVS, which coordinated the investigation through the Nosocomial Infection Early Warning, Investigation and Surveillance Network (Réseau d'Alerte, d'Investigation et de Surveillance des Infections Nosocomiale [RAISIN]), a partnership between InVS and CCLIN.

Microbiologic Investigation

All isolates of *A. baumannii* were recovered from routine clinical specimens (from blood and catheters, urine, respiratory tract, skin, and wounds) and from colonization samples (from axillary, pharyngeal, or rectal swabs), identified by standard techniques at local laboratories, then sent for confirmatory testing to a central laboratory (University Hospital of Bicêtre, K.-Bicêtre, France).

Identification was confirmed by using the API 32GN system (bioMérieux, Marcy-l'Etoile, France). *A. baumannii* strains were also tested for the ability to grow at 44°C in Trypticase soy broth (Oxoid, Unipath Ltd, Basingstoke, UK).

Routine antibiograms were determined by the disk diffusion method on Mueller-Hinton agar (BioRad, Marnes-La-Coquette, France) and interpreted as recommended by the Clinical and Laboratory Standards Institute (formerly NCCLS) (*24*). The presence of ESBL was shown by a synergy image created by using the double-disk synergy test with cefepime, ceftazidime, and ticarcillin-clavulanic acid disks on Mueller-Hinton agar plates (*16,18*). Synergy images were best seen when plates were incubated at 25°C. Alternatively, double-disk synergy tests were also performed on plates containing cloxacillin (200 μg/mL) (*16*). Analytic isoelectric focusing was performed with an ampholine polyacrylamide gel (*18*).

Molecular Investigation

Genomic DNA and plasmid extractions and electroporation of plasmid extracts into *Escherichia coli* DH10B were performed (*16*). Half of *A. baumannii* isolates from the 4 largest hospitals of 2 districts were randomly selected

during the outbreak; all isolates from smaller hospitals or from other districts were analyzed by pulsed-field gel electrophoresis (PFGE), using *Apa*I (Amersham Biosciences, Les Ulis, France) (*4*). *Apa*I-macrorestriction patterns were digitized and analyzed with Taxotron software (Institut Pasteur, Paris, France) and interpreted according to Tenover et al. (*25*).

PCR-based amplification of class 1 integron structures and of bla_{VEB-1} gene and subsequent sequencing of the bla_{VEB-1} gene were performed as described (*19*). Automated sequencing reactions were performed with the same bla_{VEB-1}-specific primers (ABI Prism 3100; Applied Biosystems, Les Ullis, France).

Results

Epidemiologic Investigation

From April 1, 2004, to June 30, 2004, 53 hospitals (41 tertiary care and 12 long-term care facilities) located in 15 districts reported 290 probable cases, of which 275 (95%) were confirmed. Of the 290 probable cases, 255 (88%) were reported through 116 mandatory notifications, and 35 (12%) were identified through strains directly sent for characterization without notification. The 2 first notifications occurred at the end of July (2 clusters totaling 15 cases) and were followed by 3 other notifications in early September (3 clusters totaling 8 cases). The monthly number of reported cases peaked in October 2003 and again in January 2004 (after intense media coverage of the outbreak); it gradually declined after this date, until the alert was canceled in June 2004 (Figure 1).

Cases were first reported in the Nord district and in 4 other contiguous districts, then later in 10 districts in 4 noncontiguous regions. Most reporting hospitals and cases were in 2 adjacent districts (59 and 62 on Figure 2A). The date of case diagnosis indicated that the strain had been circulating since April 2003 in 5 districts of northern France before the outbreak was recognized (Table 1).

Spread of this multidrug-resistant strain was mediated by large referring hospitals. Among 116 notifications, 20% came from regional teaching hospitals, 45% from public general hospitals, 15% from smaller private hospitals, and 20% from long-term care facilities; most affected wards were intensive care units (54 notifications, 47%), medical wards (55 notifications, 47%), reeducation and long-term care wards (24 notifications, 21%), and surgical wards (13 notifications, 11%). In 3 regions (northern France, Toulouse-Montpellier, Lyon), investigations suggested that frequent patient transfers between hospitals within the same healthcare network could explain the diffusion of the strain (*4,26*). In northern France (Nord and Pas de Calais districts), among 33 hospitals reporting ≥1 cases, 22 (67%) had an index case directly admitted from a previously affected facility (Figure 2B); however, no such link could be established from 1 region to another.

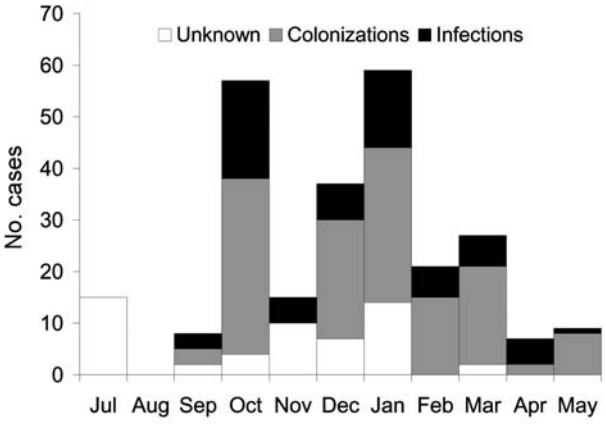

Figure 1. Number of VEB-1–producing *Acinetobacter baumannii* cases, by month of report, France, July 2003–May 2004 (N = 255).

Of the 217 (71%) cases with clinical documentation, 73 (33%) were infections and 144 (67%) were colonizations. The sources of the clinical isolates were as follows: respiratory tract (33%), skin and wounds (33%), urine (21%), catheters and blood (8%), and others (5%). By the time of notification, 34 (12%) patients had died; however, investigations in northern France suggested that only 17% of the reported deaths were related to the *A. baumannii* infection.

At several participating hospitals, environmental surfaces were swabbed for culture and found to be positive for VEB-1-producing *A. baumannii*; the organism was particularly prevalent on bed rails and respiratory equipment (data not shown) and, at 1 hospital, was also found on blood pressure cuffs (*27*).

Microbiologic Investigation

The antibiogram of VEB-1 ESBL–producing *A. baumannii* strains was similar for 275 (95%) of the 290 probable cases; the level of resistance to aminoglycosides varied slightly. A synergy image, signature of the presence of an ESBL, could not be observed between clavulanic acid and cefepime or ceftazidime disks on a routine antibiogram (Figure 3A) unless cloxacillin-containing plates that inhibit cephalosporinase activity were used (Figure 3B). Alternatively, incubation of the antibiogram at room temperature enhanced the identification of the synergy image (Figure 3C, D). One strain isolated in the Alpes-Maritimes district, next to Nice, was also resistant to colistin. All strains remained susceptible to imipenem.

Of the 288 *A. baumannii* isolates, 275 (95%) contained the bla_{VEB-1} gene, according to PCR analysis, which shows a perfect correlation between the antibiogram and the PCR results. All but 2 notifying hospitals isolated the outbreak

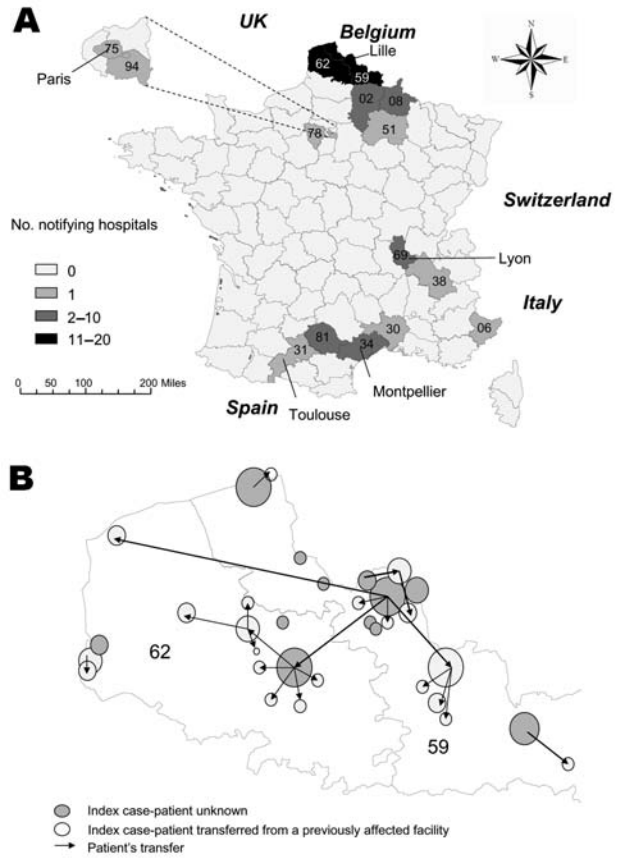

Figure 2. A) Hospitals reporting VEB-1–producing *Acinetobacter baumannii*, by district, France, April 2003–May 2004 (N = 53). Each district is identified with a number (same numbers used in Table 1). B) Interhospital spread in northern France. Circles represent affected hospitals; the sizes are proportional to the number of reported cases.

strain in their wards; for 1 hospital in Isère and 1 in Tarn, the isolate was not available for confirmatory testing. Genotyping by PFGE showed that all VEB-1 *A. baumannii* isolates were clonally related to each other and to the strain responsible for the 2001 outbreak (strain AYE [*16*]). Although most strains belonged to several subtypes of a given type, differing by only 1 or 2 bands, several strains were more distantly related and belonged to a different type. However, these isolates could be related to the main epidemic strains as illustrated on the dendrogram (Figure 4). The epidemic strain isolated in the southern part of the country differed from those in the northern part by at least 3 bands, and thus forms a separate cluster.

The sequence of $bla_{\text{VEB-1}}$ gene and its genetic environment were identical to those previously published for *A. baumannii* strain AYE (*16,18*). Finally, the chromosomal position of the integron was verified for 20 isolates randomly chosen in the different regions.

Control Measures

Recommendations for surveillance and control were disseminated to all hospitals (through specific postings on the InVS Web site and email from CCLIN to infection control units and laboratories) and then implemented by infection control units. Based on international guidelines (*28*, Table 2), the recommendations included the usual standard and contact precautions for limiting the spread of this pathogen within a hospital but added systematic screening in wards at high risk (e.g., intensive care units) and appropriate antimicrobial drug use. In addition, specific recommendations were dispatched to limit the spread of the strain from 1 hospital to another; hospitals were asked to report cases to local health departments and CCLIN, to limit patient admissions, and to inform other hospitals about infected or colonized patients before transferring them (2 hospitals even closed their wards to new patients).

Discussion

This is the first report of a clonal ESBL-producing *A. baumannii* outbreak that was nationwide. It was traced in 53 hospitals, initially in northern France and later in 4 distant regions. Recognition of the outbreak and effective tracing of new cases was facilitated by several factors. First, in August 2001, a national nosocomial infection notification system based on specific reporting criteria, including unusual antimicrobial drug resistance profiles, was implemented in France (*23*). The system relies on hospital infection control units and is coordinated at the regional level by CCLIN created in 1992 (*22*) and at the national level by InVS, which enables events of national importance to be detected. Second, the French healthcare system is organized around large public, university, or regional tertiary care hospitals that serve an entire region. These hospitals include medical microbiology laboratories that are well connected to other laboratories in smaller public hospitals, therefore enabling prompt dissemination of laboratory guidelines. Third, the outbreak strain had a unique susceptibility profile that enabled effective screening of *A. baumannii* strains in hospital laboratories and referral to a central laboratory for confirmatory testing. This laboratory provided immediate feedback (<48 hours after strains were received) to hospitals, which facilitated prompt adaptation of local control measures, and to CCLIN and InVS, which enabled regular tracing of the strain dissemination and adaptation of recommendations.

Two factors make emergence of panresistant isolates through mutations in porins (*11*) or acquisition of plasmid-encoded carbapenemases (*8*), such as the $bla_{\text{OXA-58}}$ gene (*28*), a concern. First, there may be no option but to treat patients infected with *A. baumannii*, particularly in the intensive care setting, because *A. baumannii* infection is associated with a higher case-fatality ratio in critically ill

Table 1. VEB–1-producing *Acinetobacter baumannii* case characteristics and period of transmission, by district, France, April 2003–May 2004 (N = 290)

District*	Reporting hospitals (n)	Reported cases (n)†				Reported deaths (N)	Date of case diagnosis	
		Total	I	C	U		First	Last
Nord (59)	19	124	19	60	45	24	22 Apr 2003	13 May 2004
Pas de Calais (62)	14	111	32	55	24	7	9 Mar 2003	14 May 2004
Aisne (02)	2	2	0	0	2	0	5 Nov 2003	5 Nov 2003
Ardennes (08)	2	14	6	8	0	0	15 May 2003	2 Feb 2004
Champagne (51)	1	11	2	9	0	1	13 Aug 2003	15 Mar 2004
Paris (75)	1	1	1	0	0	0	31 Jan 2004	31 Jan 2004
Val de Marne (94)	1	1	1	0	0	0	3 May 2004	3 May 2004
Yvelines (78)	1	1	0	1	0	0	8 Mar 2004	8 Mar 2004
Rhône (69)	4	8	4	4	0	0	13 Aug 2003	7 Feb 2004
Isère (38)	1	1	0	1	0	0	2 Dec 2003	2 Dec 2003
Haute Garonne (31)	1	5	1	2	2	1	16 Sep 2003	15 Apr 2004
Tarn (81)	2	2	1	1	0	0	28 Oct 2003	5 Jan 2004
Hérault (34)	2	7	5	2	0	1	21 Oct 2003	9 Apr 2004
Gard (30)	1	1	1	0	0	0	10 Apr 2003	10 Apr 2004
Alpes Maritimes (06)	1	1	0	1	0	0	3 Jan 2004	3 Jan 2004
Total	53	290	73	144	73	34	22 Apr 2003	14 May 2004

*Districts are listed from north to south and west to east; numbers are those used in Figure 2 to identify districts.
†I, infected; C, colonized; U, unknown.

patients (*3*,*4*,*29*,*30*). Second, carbapenems were the last molecules active against *A. baumannii* VEB-1 isolates. Antimicrobial drug susceptibility of *A. baumannii* VEB-1 isolates remained relatively stable; all tested strains were fully susceptible to carbapenems and all but 1 was susceptible to colistin. The slight variations in their aminoglycoside susceptibilities reflected presumably different antimicrobial drug selection pressures in some of the hospitals.

Early warning and investigations of reported cases alerted all hospitals of the need for rapid identification and reporting of cases. Furthermore, early warning enabled timely assistance for implementing effective control measures. These investigations showed that the epidemic clone was already endemic in some hospitals, which suggests that once the strain is introduced into a hospital, eradicating it may be difficult. Early recognition of its presence and prompt implementation of strict infection control measures are therefore necessary to prevent its further spread and establishment of endemicity. Moreover, when several hospitals in the same network are affected by the same clone, coordinated measures must be implemented to effectively reduce its spread, as demonstrated for other multidrug-resistant bacteria such as vancomycin-resistant enterococci (*31*).

In this national investigation, spread within hospitals was not explored because it has been well described in the literature (*3*,*4*,*26*,*27*). While we reinforced the implementation of standard and contact precautions in affected hospitals, we recommended that other hospitals be informed (by flagged records) when infected or colonized patients are transferred to them. In some hospitals, fast-spreading

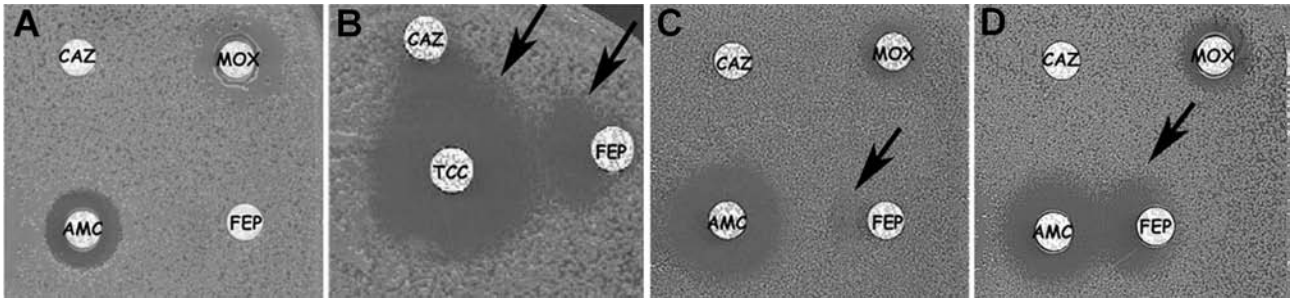

Figure 3. Extended-spectrum β-lactamase (ESBL) laboratory identification. Usefulness of double-disk synergy test with bla_{VEB-1}-positive *Acinetobacter baumannii* strain on Mueller-Hinton agar plates with clavulanate as inhibitor. The disks tested contained ticarcillin + clavulanate (TCC), amoxicillin + clavulanate (AMC), moxalactam (MOX), ceftazidime (CAZ), and cefepime (FEP). A) Standard disk diffusion as recommended by Clinical and Laboratory Standards Institute at 37°C (98°F). B) Standard disk diffusion on cloxacillin-containing Mueller-Hinton plates at 37°C (98°F). Cloxacillin inhibits partially the naturally occurring cephalosporinase (AmpC) from *A. baumannii*, thus enabling easier detection of possible ESBL phenotypes. C) Standard disk diffusion at 25°C (77°F). D) Standard disk diffusion at 25°C (77°F) when AMC and FEP disks were brought closer. The presence of ESBL was shown by a synergy image, as indicated with the arrows. ESBL presence was best seen on cloxacillin-containing (B) plates or at reduced growth temperature (D).

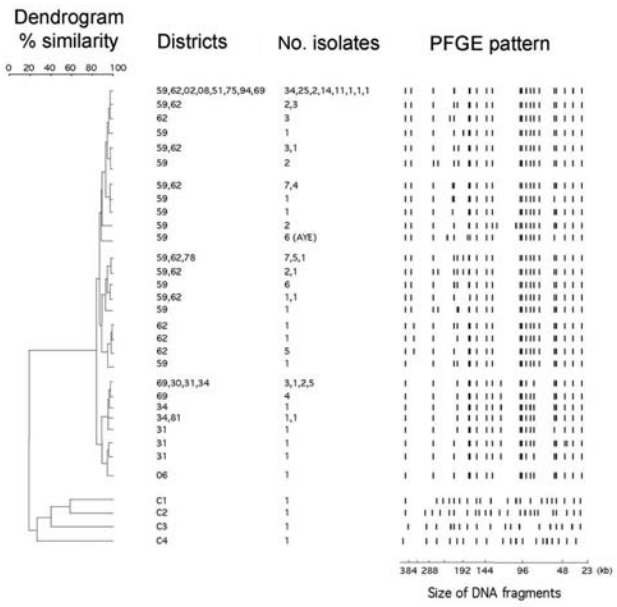

Figure 4. Digitized pulsed-field gel electrophoresis (PFGE) patterns and phylogenetic tree of 183 VEB-1–producing *Acinetobacter baumannii* isolates. Half of *A. baumannii* isolates from the 4 largest hospitals of 2 districts (59 and 62) were randomly selected over the entire epidemic period; all isolates from smaller hospitals or from other districts were included. The PFGE pattern of the *A. baumannii* AYE reference strain previously described is indicated in brackets (*16*). *Apa*I macrorestriction patterns were digitized and analyzed with Taxotron software (Institut Pasteur, Paris, France) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair-group method using arithmetic averages clustering. The scale indicates the level of pattern similarity. PFGE results were interpreted according to the criteria of Tenover et al (*25*). For a given PFGE pattern, the districts, along with the number of times a given PFGE pattern was found, are also indicated.

clusters associated with deaths led to the closure of intensive care units; however, the effectiveness of such a measure needs further evaluation because transfer of these patients enhances the dissemination of the strain (*4*). In other hospitals, the prompt and strict application of barrier precautions, without closure of the intensive care unit, effectively controlled the outbreak.

Clones may emerge at different locations by independent selection of genetically related, circulating strains in the community or environment as a result of antibiotic use in hospitals (*2*,*8*,*32*,*33)*. Otherwise, similar isolates may appear at different locations simply through direct spread from 1 hospital to another. Results of this investigation suggest that regional spread of the organism was mediated by patient transfers within regional healthcare networks. However, the appearance of the organism in hospitals in southern France, Lyon (central France), and Paris is difficult to explain by simple spread. Although *A. baumannii* isolates from southern and northern France were found in Lyon, no epidemiologic link could be found, and one cannot be sure that the cases in southern France were acquired from contact with patients or hospitals in northern France. Case reporting and recognition of the outbreak could have been delayed, and the epidemic situation that we observed might be the consequence of a spread that started earlier than 2003, even earlier than the 2001 outbreak in Valenciennes (*4*). Several facts support this hypothesis: 1) $bla_{\text{VEB-1}}$ gene was characterized in 1996 in an *E. coli* strain isolated from a Vietnamese child hospitalized in France (*18*) and then in 2001 in an *A. baumannii* strain (*16*); 2) the 2001 *A. baumannii* isolates were clonally related, but at least 2 PFGE types had already been observed (*4*); 3) retrospective surveys recently conducted in a few large university hospitals in southern and northern France showed that *A. baumannii* VEB-1 isolates were present as early as January 2001 (data not shown). Finally, an alternative explanation could be that the isolates from northern and southern France were introduced separately into the country from an unknown common source.

The origin of this clone remains unknown because *A. baumannii* VEB-1 isolates have never been reported in other countries. All isolates were epidemiologically related, and most of them were similar enough to be considered as belonging to the same strain. However, the diversity of the total set of isolates was slightly greater than what is usually recovered in single-hospital outbreaks. Clonal diffusion with several pulsotypes has already been observed in the first *A. baumannii* VEB-1 outbreak described in 2001 (*4*). That isolates with indistinguishable PFGE profiles were found in many of the hospitals and that all isolates had similar profiles suggest that they could be considered relatively new, compared with older strains circulating in Europe (*32*). Although the origin of $bla_{\text{VEB-1}}$ gene is presumably countries in Southeast Asia, it would be interesting to investigate the occurrence of such strains in these countries. Alternatively, a European *A. baumannii* strain might have acquired foreign DNA containing $bla_{\text{VEB-1}}$ through either conjugation or transformation (*34*). Several multidrug-resistant *A. baumannii* strains have been found to be widespread in Europe (*32*,*33*), and epidemic carbapenem-resistant strains have been reported worldwide (*9*,*35*–*37*).

This report used data only from mandatory notifications and isolates received by the central laboratory; the full extent of this clone in France remains unknown. The large media coverage of this outbreak in late December 2003 may have discouraged hospitals from reporting cases; several isolates were actually sent to the central laboratory without being reported. Moreover, because mandatory notification is not patient-based, contact tracing of

Table 2. Recommendations for hospitals*†

Type	Recommendation
Notification	Report any case of infection or colonization with *Acinetobacter baumannii* strain showing resistance profile similar to that of 2001 outbreak strain (*16*) to CCLIN and local health department; attach copy of antibiogram.
Laboratory guidelines	Establish identification criteria based on antibiogram
	Store strains and contact central laboratory for microbiologic investigation
Medical wards	Inform all medical teams and paramedics of presence of bacterial strain
	Ensure appropriate use of antimicrobial drugs in high-risk wards
Infection control team	Set up systematic screening of patients in high-risk wards
	Reinforce isolation protocols and standard hygiene precautions throughout hospital
	Reinforce surface cleaning procedures in wards where infected or colonized patients have been
Patient transfer	Limit internal and external patient transfers
	Inform receiving hospitals of status of patients colonized or infected with ESBL-producing *A. baumannii*

*CCLIN, regional infection control coordinating centers; ESBL, extended-spectrum β-lactamase.
†At the national level, recommendations were disseminated by the National Institute of Public Health (Institut de Veille Sanitaire) and the French Nosocomial Infection Early Warning, Investigation and Surveillance Network (Réseau d'Alerte, d'Investigation et de Surveillance des Infections Nosocomiales) through its website (http://www.invs.sante.fr/raisin, "alerte" section). At the regional level, they were disseminated to all hospital laboratories and infection control units by CCLIN through email notifications and their respective websites. In each hospital, they were implemented in the wards by infection control units assisted by CCLIN, when necessary.

each patient could not be systematically performed. Data from notifications (date of first and last case, name of transferring facility for imported cases) enabled us to establish only when an index patient was admitted from a previously affected facility.

Because European countries were informed about this outbreak through the EU Early Warning and Response System, Belgian public health authorities were able to detect early and control a cluster of 3 cases in a nursing home close to the border with France (*38*). This clone should therefore be considered as an issue affecting hospitals not only in France but also in bordering countries, and this situation underlines the importance of supranational information exchange for early warning of antimicrobial drug resistance.

Conclusion

The emergence and spread of this strain of VEB-1–producing *A. baumannii* isolates are worrisome and reflect the magnitude of antimicrobial drug resistance in France. Most of the reported cases occurred in northern France. The weekly number of reported cases dropped substantially after January 2004, which suggests that infection control recommendations were effective. After the alert was stopped in June 2004, only 12 notifications were received by InVS until December 2005; these notifications came from 8 hospitals (5 of them already known) and indicated a total of 17 VEB-1–producing *A. baumannii* infections. The last notification was received in May 2005, suggesting that the national outbreak was controlled. However, after June 2004 the notification system returned to its baseline setting; i.e., only multidrug-resistant *A. baumannii* infections were to be reported. Although we are confident that the national alert drastically helped reduce the clinical effect of the outbreak, we cannot rule out that the strain is still spread by colonized patients.

This study emphasizes the importance of an early warning network comprising infection control units and regional (CCLIN, health departments) and central (InVS and expert laboratory) structures. The results underscore the need for anticipating future, emerging antimicrobial drug resistance threats by combining laboratory and epidemiologic expertise. Early detection of emerging resistance mechanisms usefully completes surveillance data, which monitor the level of resistance but may miss the emergence of new phenomena.

Acknowledgments

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References

1. Allen DM, Hartman BJ. *Acinetobacter* species. In: Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. 5th ed. Philadelphia: Churchill Livingstone; 2000:2339–44.
2. Bergogne-Bérézin E, Towner KJ. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical and epidemiological features. Clin Microbiol Rev. 1996;9:148–65.
3. Villegas MV, Hartstein AI. *Acinetobacter* outbreaks, 1977–2000. Infect Control Hosp Epidemiol. 2003;24:284–95.

4. Carbonne A, Naas T, Blanckaert K, Couzigou C, Cattoen C, Aggoune M, et al. Investigation of a nosocomial outbreak of extended-spectrum β-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a hospital setting. J Hosp Infect. 2005;60:14–8.
5. Réseau d'alerte, d'investigation et de surveillance des infections nosocomiales. Enquête nationale de prévalence 2001—Résultats. Saint-Maurice (France): Institut de Veille Sanitaire; 2003. p. 22 [cited 2005 Sep 27]. Available from http://www.invs.sante.fr/publications/2003/raisin_enp_2001/raisin_enp_2001_p_19_50.pdf
6. Gales AC, Jones RN, Forward KR, Linares J, Sader HS, Verhoef J. Emerging importance of multidrug-resistant *Acinetobacter* species and *Stenotrophomonas maltophilia* as pathogens in seriously ill patients: geographic patterns, epidemiological features, and trends in the SENTRY Antimicrobial Surveillance Program (1997–1999). Clin Infect Dis. 2001;32:S104–13.
7. Afzal-Shah M, Livermore DM. Worldwide emergence of carbapenem-resistant *Acinetobacter* spp. J Antimicrob Chemother. 1998;41:576–7.
8. Nordmann P, Poirel L. Emerging carbapenemases in gram-negative aerobes. Clin Microbiol Infect. 2002;8:321–31.
9. Landman D, Quale JM, Mayorga D, Adedeji A, Vangala K, Ravishankar J, et al. Citywide clonal outbreak of multiresistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in Brooklyn, NY. Arch Intern Med. 2002;162:1515–20.
10. Bou G, Cerveró G, Domínguez MA, Quereda C, Martínez-Beltrán J. Characterization of a nosocomial outbreak caused by a multiresistant *Acinetobacter baumannii* strain with a carbapenem-hydrolyzing enzyme: high-level carbapenem resistance in *A. baumannii* is not due solely to the presence of β-lactamases. J Clin Microbiol. 2000;38:3299–305.
11. Fernández-Cuenca F, Martínez-Martínez L, Conejo MC, Ayala JA, Perea EJ, Pascual A. Relationship between β-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. J Antimicrob Chemother. 2003;51:565–74.
12. Corvec S, Caroff N, Espaze E, Giraudeau C, Drugeon H, Reynaud A. AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. J Antimicrob Chemother. 2003;52:629–35.
13. Vahaboglu H, Öztürk R, Aygün G, Coskunkan F, Yaman A, Kaygusuz A, et al. Widespread detection of PER-1-type extended-spectrum β-lactamases among nosocomial *Acinetobacter* and *Pseudomonas aeruginosa* isolates in Turkey: a nationwide multicenter study. Antimicrob Agents Chemother. 1997;41:2265–9.
14. Yong D, Shin JH, Kim S, Lim Y, Yum JH, Lee K, et al. High prevalence of PER-1 extended-spectrum beta-lactamase–producing *Acinetobacter* spp. in Korea. Antimicrob Agents Chemother. 2003;47:1749–51.
15. Poirel L, Karim A, Mercat A, Le Thomas I, Vahaboglu H, Richard C, et al. Extended-spectrum β-lactamase-producing strain of *Acinetobacter baumannii* isolated from a patient in France. J Antimicrob Chemother. 1999;43:157–65.
16. Poirel L, Menuteau O, Agoli N, Cattoen C, Nordmann P. Outbreak of extended-spectrum β-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a French hospital. J Clin Microbiol. 2003;41:3542–7.
17. Nagano N, Nagano Y, Cordevant C, Shibata N, Arakawa Y. Nosocomial Transmission of CTX-M-2 β-lactamase–producing *Acinetobacter baumannii* in a neurosurgery ward. J Clin Microbiol. 2004;42:3978–84.
18. Poirel L, Naas T, Guibert M, Chaibi EB, Labia R, Nordmann P. Molecular and biochemical characterization of VEB-1, a novel class A extended-spectrum beta-lactamase encoded by an *Escherichia coli* integron gene. Antimicrob Agents Chemother. 1999;43:573–81.
19. Naas T, Mikami Y, Imai T, Poirel L, Nordmann P. Characterization of In53, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. J Bacteriol. 2001;183:235–49.
20. Girlich D, Naas T, Leelaporn A, Poirel L, Fennewald M, Nordmann P. Nosocomial spread of the integron-located veb-1-like cassette encoding an extended-spectrum beta-lactamase in *Pseudomonas aeruginosa* in Thailand. Clin Infect Dis. 2002;34:603–11.
21. Horan TC, Gaynes RP. Surveillance of nosocomial infections. In: Mayhall CG, editor. Hospital epidemiology and infection control. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2004. p. 1659–702.
22. Astagneau P, Brucker G. Organization of hospital-acquired infection control in France. J Hosp Infect. 2001;47:84–7.
23. Coignard B, Lepoutre A, Desenclos JC. Lessons learned from implementing a mandatory notification of hospital acquired infections in France [cited June 11, 2006]. HELICS Conference; Nov 27, 2004; Lyon, France. Available from http://helics.univ-lyon1.fr/conference/6a.pdf
24. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; fifteenth informational supplement. M100–S15. Wayne (PA): The Institute; 2005.
25. 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.
26. Carbonne A, Blanckaert K, Naas T, Seringe E, Botherel AH, Aggoune M, et al. Investigation et contrôle d'une épidémie régionale à *Acinetobacter baumannii* producteur de bêta-lactamase à spectre étendu VEB-1, Nord—Pas de Calais, avril 2003 à février 2004. Bulletin Epidémiologique Hebdomadaire [serial online]. 2004;32–3 [cited 2005 May 5]. Available from http://www.invs.sante.fr/beh/2004/32_33/index.htm
27. Bureau-Chalot F, Drieux L, Pierrat-Solans C, Forte D, de Champs C, Bajolet O. Blood pressure cuffs as potential reservoirs of extended-spectrum beta-lactamase VEB-1-producing isolates of *Acinetobacter baumannii*. J Hosp Infect. 2004;58:91–2.
28. Hérìtier C, Dubouix A, Poirel L, Marty N, Nordmann P. A nosocomial outbreak of *Acinetobacter baumannii* isolates expressing the carbapenem-hydrolysing oxacillinase OXA-58. J Antimicrob Chemother. 2005;55:115–8.
29. Shlaes DM, Gerding DN, John JFJ, Craig WA, Bornstein DL, Duncan RA, et al. Society for Healthcare Epidemiology of America and Infectious Diseases Society of America Joint Committee on the Prevention of Antimicrobial Resistance: guidelines for the prevention of antimicrobial resistance in hospitals. Infect Control Hosp Epidemiol. 1997;18:275–91.
30. Garcia-Garmendia JL, Ortiz-Leyba C, Garnacho-Montero J, Jimenez-Jimenez FJ, Monterrubio-Villar J, Gili-Miner M. Mortality and the increase in length of stay attributable to the acquisition of *Acinetobacter* in critically ill patients. Crit Care Med. 1999;27:1794–9.
31. Ostrowsky BE, Trick WE, Sohn AH, Quirk SB, Holt S, Carson LA, et al. Control of vancomycin-resistant *Enterococcus* in health care facilities in a region. N Engl J Med. 2001;344:1427–33.
32. van Dessel H, Dijkshoorn L, van der Reijden T, Bakker N, Paauw A, van den Broek P, et al. Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals. Res Microbiol. 2004;155:105–12.
33. Marque S, Poirel L, Heritier C, Brisse S, Blasco MD, Filip R, et al. Regional occurrence of plasmid-mediated carbapenem-hydrolyzing oxacillinase OXA-58 in *Acinetobacter* spp. in Europe. J Clin Microbiol. 2005;43:4885–8.
34. de Vries J, Wackernagel W. Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. Proc Natl Acad Sci U S A. 2002;99:2094–9.
35. Turton JF, Kaufmann ME, Warner M, Coelho J, Dijkshoorn L, van der Reijden T, et al. A prevalent, multiresistant clone of *Acinetobacter baumannii* in southeast England. J Hosp Infect. 2004;58:170–9.

36. Dalla-Costa LM, Coelho JM, Souza HA, Castro ME, Stier CJ, Bragagnolo KL, et al. Outbreak of carbapenem-resistant *Acinetobacter baumannii* producing the OXA-23 enzyme in Curitiba, Brazil. J Clin Microbiol. 2003;41:3403–6.
37. Naas T, Levy M, Hirschauer C, Marchandin H, Nordmann P. Outbreak of carbapenem-resistant *Acinetobacter baumannii* producing the carbapenemase OXA-23 in a tertiary care hospital of Papeete, French Polynesia. J Clin Microbiol. 2005;43:4826–9.
38. Jans B, Glupczynski Y, Suetens C, Van Cleemput E. Enquête épidémiologique relative à *Acinetobacter baumannii* producteur de BLSE (type VEB-1) en Belgique. Institut Scientifique de la Santé Publique, ISP/EPI Report No. 2004–18, Octobre 2004 [cited 2005 Sep 27]. Available from http://www.iph.fgov.be/epidemio/epinl/nsi-hnl/acinetobacter.pdf

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Macrolide Resistance in Adults with Bacteremic Pneumococcal Pneumonia

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We conducted a case-control study of adults with bacteremic pneumococcal pneumonia to identify factors associated with macrolide resistance. Study participants were identified through population-based surveillance in a 5-county region surrounding Philadelphia. Forty-three hospitals contributed 444 patients, who were interviewed by telephone regarding potential risk factors. In multivariable analyses, prior exposure to a macrolide antimicrobial agent (odds ratio [OR] 2.8), prior flu vaccination (OR 2.0), and Hispanic ethnicity (OR 4.1) were independently associated with an increased probability of macrolide resistance, and a history of stroke was independently associated with a decreased probability of macrolide resistance (OR 0.2). Fifty-five percent of patients with macrolide-resistant infections reported no antimicrobial drug exposure in the preceding 6 months. Among patients who reported taking antimicrobial agents in the 6 months preceding infection, failure to complete the course of prescribed drugs was associated with an increased probability of macrolide resistance (OR 3.4).

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia in adults. Bacteremic pneumococcal pneumonia is among the most serious forms of pneumococcal disease, and incidence rises steeply with advancing age (*1*). Although considerable controversy exists about the clinical impact of pneumococcal drug resistance (*2*), the prevalence of single-drug and multidrug–resistant pneumococci has increased in the last 2 decades (*3*,*4*). Drug-resistant pneumococci clearly emerge under the selective forces of antibacterial drugs used in the population. Still, the precise nature of these selection mechanisms and the risk associated with different types of exposures are not well defined.

Pneumococcal resistance to macrolides is a problem because macrolides are among the most common oral drugs used to treat patients with community-acquired pneumonia (*5*). A recent study found that patients with macrolide-resistant pneumococcal bacteremia were substantially more likely to have been exposed to macrolide therapy before hospitalization than were patients with macrolide-susceptible pneumococcal bacteremia (*6*). Since most initial therapy of community-acquired pneumonia is empiric, estimating the probability of macrolide-resistant pneumococcal disease is necessary to select appropriate therapy. Indeed, current treatment guidelines recommend not prescribing macrolide therapy alone for patients with community-acquired pneumonia if they report exposure to macrolides within the 3 months preceding the onset of illness (*7*).

We conducted a population-based case-control study to identify clinical and demographic factors independently associated with macrolide-resistant bacteremic pneumococcal pneumonia in adults. We used a detailed multistage interview method to elicit in-depth histories of exposure to antimicrobial agents to examine whether disease probability varied across different patterns of antibacterial drug exposure.

Methods

Design

We conducted a case-control study within a network of hospitals conducting prospective population-based surveillance for bacteremic pneumococcal pneumonia in adults in southeastern Pennsylvania from December 1,

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2000, to April 17, 2004 (Appendix). Patients were all hospitalized adults with macrolide-resistant bacteremic pneumococcal pneumonia, and controls were all hospitalized adults with macrolide-susceptible bacteremic pneumococcal pneumonia.

Study Site

This study was conducted within the 5-county region surrounding Philadelphia, Pennsylvania: Bucks, Chester, Delaware, Montgomery, and Philadelphia Counties. The adult population (age ≥18 years) of this region is 2,881,132 (US Census 2000). At the start of the surveillance period, 46 acute-care hospitals served this region; 43 of them participated in this study. Of the remaining 3 hospitals, 2 were small hospitals closed to external studies, and 1 was a larger academic hospital that was unable to participate.

Study Participants

Inclusion criteria for the study were persons who 1) were ≥18 years of age, 2) had at least 1 blood culture that grew *S. pneumoniae* drawn within 48 hours of hospital admission, 3) resided in 1 of the 5 counties, and 4) had a bacterial isolate confirmed in our laboratory as *S. pneumoniae* (see below). Study participants were further restricted on the basis of physician report to those patients with radiographic evidence of an acute respiratory infection. Exclusion criteria for the case-control study included evidence of bacterial meningitis (cerebrospinal fluid [CSF] growth of *S. pneumoniae* or CSF findings compatible with bacterial meningitis) or hospitalization within 10 days preceding the index hospitalization. Study participants who died during hospitalization were included in this study; information about them was collected by interviewing a suitable proxy respondent.

Study participants were identified by microbiology laboratory personnel at each participating hospital. Whenever laboratory personnel identified a blood culture with growth of *S. pneumoniae*, research staff contacted the physician of record to determine the patient's eligibility. Eligible participants (or proxies in cases of mental incompetence or death) were then approached for study enrollment at a time determined by the treating physician (typically after hospital discharge). Participants were mailed informational study materials and then contacted by phone to provide consent for study participation and complete a telephone interview.

Data Collection

Trained telephone interviewers completed a telephone interview with each study participant that covered demographic and clinical areas. Questions focused on the demographic and clinical status of the patient immediately before hospitalization for pneumococcal pneumonia. A multistep strategy was used to obtain the most complete drug histories possible. A phased approach was employed in which the interviewer first asked open-ended questions about use of drugs, then asked indication-specific questions about medications used (e.g., for respiratory tract infections, urinary tract infection) and, finally, named antimicrobial drugs by brand and generic names while the participant referred to photo hand cards (mailed to the participants in advance) that displayed the study drugs of interest. In prior research, including a study of antimicrobial drug recall, each of these steps has dramatically increased drug recall (*8–11*). Participants were asked to distinguish antimicrobial agents that were being taken at the time of hospitalization from those drugs that were taken for illnesses preceding the onset of pneumococcal pneumonia. We focused on patient self-report of prior antimicrobial drug exposure to mimic the information that would be available at the time of diagnosis and empiric treatment decisions. However, we also contacted the primary care physicians of study participants to obtain documentation of antimicrobial agents prescribed in the 6-month period preceding the hospitalization for pneumococcal pneumonia. In addition, since study participants were interviewed at home, we asked them to examine any medication bottles that they still possessed to verify the name of the drug and to determine if any medications were unfinished.

Microbiologic Data Collection

Pneumococcal blood isolates were transported to a central laboratory at the Hospital of the University of Pennsylvania for analysis. Isolates were re-identified to confirm that they were pneumococci on the basis of colony shape and hemolytic activity, Gram stain appearance, catalase reaction, bile solubility, and optochin susceptibility (*12*).

Confirmed isolates of *S. pneumoniae* were screened for susceptibility to oxacillin, erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole, and levofloxacin by using the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards) disk-diffusion procedure (*13*). Isolates that demonstrated reduced susceptibility to any drug were confirmed by using a Food and Drug Administration–cleared and CLSI-compliant microbroth dilution testing method (Sensititer 96 well plate, Trek Diagnostics Systems, Inc., Cleveland, OH, USA) for *S. pneumoniae*. Because the highest erythromycin microbroth MICs that could be measured with the assay were 4 μg/mL, additional testing was performed on all erythromycin-resistant isolates with the Etest method, as recommended by the manufacturer, which includes the use of Mueller-Hinton 5% sheep's blood agar (BBL brand, BD

Diagnostic Systems, Sparks, MD, USA) and incubation in 5% CO_2 for 20 to 24 h. Carbon dioxide incubation, required for the optimal growth of many pneumococci on solid media, increases erythromycin MICs by ≈1 doubling dilution and therefore changes the erythromycin MIC breakpoints to ≤0.5, susceptible, 1 intermediate, and ≥2 resistant (*14*). For the purposes of this study, we combined isolates with intermediate susceptibility and resistance to erythromycin as erythromycin-resistant cases for the case-control study. However, only 1 isolate among these cases had an erythromycin MIC = 1.0 μg/mL; the remainder had MICs >1.0 μg/mL.

All pneumococcal isolates were serotyped according to standard methods by using the Quellung reaction (*15–17*). All sera were purchased from the Statens Serum Institut (WHO Collaborating Centre for Reference and Research on Pneumococci) and included 14 pooled sera, 62 factor sera, and 22 type sera.

Data Analysis

We calculated descriptive statistics for case-patients and controls and compared the distribution of demographic and clinical characteristics by using χ^2 test statistics. We compared the self-reported patterns of prior antimicrobial drug exposure between case-patients and controls, distinguishing antimicrobial agents that were taken before the onset of the illness and those taken during the current illness up to the time of hospitalization.

Multivariable analyses were completed with logistic regression. We included as candidate risk factors all variables that were significantly associated with case versus control status at $p<0.10$ in bivariate analyses. We developed a final model using backward elimination, with variables with $p\geq0.05$ eliminated from the model. Associations between risk factors and macrolide-resistant bacteremic pneumococcal pneumonia that remained in the model are presented as odds ratios (ORs) and 95% confidence intervals (CIs). A separate model examining patterns of antimicrobial drug exposure was constructed restricted to those participants who reported ≥1 prior exposure to antimicrobial drugs during the 6 months preceding onset of illness. This study was approved by the institutional review boards at the University of Pennsylvania School of Medicine and each participating hospital.

Results

From December 1, 2000, through April 17, 2004, a total of 1,209 cases of pneumococcal bacteremia among adults in the 5-county region were identified. Excluding patients without a concurrent diagnosis of pneumonia, with a concurrent diagnosis of meningitis, residence outside the 5-county region, or hospitalization within 10 days of the episode yielded 956 eligible participants. We enrolled 444 (46%). Reasons for nonenrollment included physician refusal (26%), patient or proxy refusal (36%), and inability to locate the patient or family (24%).

Seventy- six patients (17%) had erythromycin-resistant infections (MIC_{50} = 8.0 μg/mL, MIC_{90} = 256 μg/mL, range 1–256 μg/mL) and were selected as the case-patients for this study (Figure). As expected, 22 of 23 isolates with erythromycin MICs ≥64 μg/mL were also clindamycin resistant (MLS_B phenotype), and 49 of 53 isolates with erythromycin MICs ≤32 μg/mL were clindamycin susceptible and comprised the M phenotype (*18*). Compared to the pneumococcal isolates from patients with erythromycin-susceptible infections, isolates from patients with erythromycin-resistant infections were more likely to have reduced susceptibility to penicillin (75% vs. 11%), tetracycline (38% vs. 1%), and trimethoprim-sulfamethoxazole (62% vs. 10%) (all $p<0.0001$). However, susceptibility to fluoroquinolones (specifically levofloxacin) was the same for erythromycin-resistant and erythromycin-susceptible isolates (1% of erythromycin-susceptible and -resistant isolates were resistant to levofloxacin, $p = 0.82$). Compared to the erythromycin-susceptible isolates, erythromycin-resistant isolates were more than twice as likely to belong to 1 of the 7 serotypes contained within the new pneumococcal conjugate vaccine (45% vs. 22%, $p<0.0001$).

Demographic and Clinical Risk Factors

Among potential demographic factors, only race and ethnicity were significantly associated with erythromycin resistance. White patients were more likely to have a resistant infection compared to patients self-reporting other racial categories (Asian, African American or Black, and Native Hawaiian or other Pacific Islander) (OR 1.8, 95% CI 1.0–3.1), and Hispanic patients were more likely

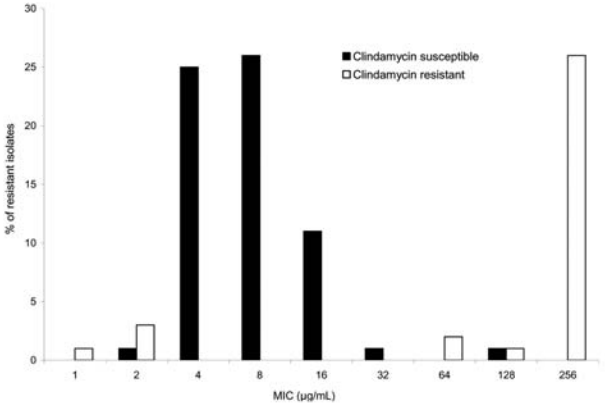

Figure. MIC distribution of resistant isolates. This figure displays the proportion of resistant isolates at each MIC. Isolates with clindamycin susceptibility are analyzed separately from isolates with clindamycin resistance. The total number of isolates is 76.

to have a resistant infection compared to non-Hispanic patients (OR 3.1, 95% CI 1.1–8.8). Patient age, sex, education, income, and urban versus suburban residence were not significantly associated with the probability of an erythromycin-resistant infection (Table 1).

Among potential clinical factors, a history of chronic bronchitis or emphysema was associated with an increased probability of erythromycin resistance (OR 2.0, 95% CI 1.1–3.4), and a history of stroke was associated with a reduced probability of resistance (OR 0.2, 95% CI 0.1–0.9). Patient report of receiving influenza vaccination in the prior year was associated with an increased probability of resistance (OR 1.7, 95% CI 1.0–2.8). Other coexisting conditions, including HIV infection, asthma, and diabetes mellitus, were not significantly associated with susceptibility of the pneumococcal isolate (Table 1).

Patient report of any exposure to antimicrobial agents during the 6 months preceding the episode of bacteremic pneumococcal pneumonia was associated with a >2-fold increase in the odds of having an erythromycin-resistant isolate (OR 2.2, 95% CI 1.3–3.7). Fifty-five percent of patients with erythromycin-resistant infections did not report any antimicrobial drug exposures in the preceding 6 months. Among different classes of antimicrobial agents, prior exposure to macrolides and quinolones was each associated with an increased probability of macrolide resistance, but reported exposure to penicillins, cephalosporins, and tetracyclines was not associated with an increased probability (Table 2). The major macrolide drugs reported by patients were azithromycin (58%) and clarithromycin (30%). The major fluoroquinolone drugs reported by patients were levofloxacin (49%) and ciprofloxacin (40%). Physicians provided outpatient medical record information on antimicrobial drug exposure for 342 (77%) of the 444 study participants. Based on physician report, documented prescription of an antimicrobial agent in the 6 months preceding the episode of bacteremic pneumococcal pneumonia was associated with an almost 2-fold increase in the odds of having an erythromycin-resistant isolate, but this finding did not achieve significance (OR 1.7 95% CI 1.0–3.1).

Among patients who reported any prior exposure to antimicrobial drugs, the probability of macrolide resistance increased with patient report of increasing number of prior courses of drugs. Patients who reported only 1 prior course had a 1.5-fold increased odds of a resistant infection, whereas patients who reported ≥2 courses of antimicrobial agents had a 3.0-fold increased odds of a resistant infection. In addition, the relationship of antimicrobial drug exposure to the probability of an erythromycin-resistant infection was time sensitive: patients with such exposure within 3 months of infection had significantly

Table 1. Demographic and clinical characteristics of cases and controls*

Characteristic	Macrolide resistant, n = 76 (%)	Macrolide susceptible, n = 368 (%)	OR	95% CI	p value
Demographic factors					
Age >65 y	40 (53)	163 (44)	1.4	0.9–2.3	0.19
Male sex	36 (48)	169 (46)	1.1	0.7–1.8	0.73
White race	57 (76)	236 (64)	1.8	1.0–3.1	0.048
Hispanic ethnicity	6 (8)	10 (2)	3.1	1.1–8.8	0.026
Nursing home residence	4 (5)	23 (6)	0.8	0.2–2.6	0.71
Annual income >US $25,000	30 (55)	136 (49)	1.2	0.7–2.2	0.46
More than high-school education	53 (72)	236 (64)	1.4	0.8–2.4	0.24
Children (≤ 6 y) in home	12 (16)	62 (17)	0.9	0.4–1.9	0.82
Philadelphia residence	29 (39)	176 (48)	0.7	0.4–1.2	0.15
Clinical factors					
HIV infection	5 (7)	45 (12)	0.5	0.2–1.3	0.17
Active smoking	25 (33)	107 (29)	1.2	0.7–2.1	0.45
Asthma	19 (25)	73 (17)	1.4	0.8–2.5	0.28
Chronic bronchitis/emphysema	23 (31)	68 (18)	2.0	1.1–3.4	0.017
Coronary artery disease	9 (12)	67 (18)	0.6	0.3–1.3	0.20
Congestive heart failure	8 (11)	72 (20)	0.5	0.2–1.1	0.069
History of stroke	2 (3)	43 (12)	0.2	0.1–0.9	0.019
Diabetes mellitus	12 (17)	89 (24)	0.7	0.4–1.3	0.20
Chronic renal disease	3 (4)	40 (11)	0.3	0.1–1.1	0.068
Active cancer	15 (20)	51 (14)	1.6	0.8–3.0	0.17
Chronic liver disease	7 (9)	43 (12)	0.8	0.3–1.8	0.56
Prior influenza vaccination†	46 (61)	178 (48)	1.7	1.0–2.8	0.039
Prior pneumococcal vaccination‡	35 (47)	198 (54)	0.8	0.5–1.2	0.27

*OR, odds radio; CI, confidence interval. p value based on χ^2 test.
†In the 12 months preceding the date of infection.
‡At any time before the date of infection.

Table 2. Patterns of prior antimicrobial drug exposure for patients and controls*

Antimicrobial agent exposure	Macrolide resistant, n = 76 (%)	Macrolide susceptible, n = 368 (%)	OR	95% CI	p value
Any in prior 6 mo†	34 (45)	101 (27)	2.2	1.3–3.7	0.002
Any macrolide in prior 6 mo	14 (19)	29 (8)	2.7	1.3–5.4	0.004
Any quinolone in prior 6 mo	14 (19)	33 (9)	2.3	1.2–4.6	0.013
Any penicillin in prior 6 mo	8 (11)	36 (10)	1.1	0.5–2.5	0.81
Any cephalosporin in prior 6 mo	4 (5)	15 (4)	1.3	0.4–4.1	0.62
Any tetracycline in prior 6 mo	1 (1)	3 (1)	1.6	0.2–16.1	0.66
No. antimicrobial agents in 6 mo					
None	41 (55)	261 (71)	Referent		
1 prescription	18 (24)	75 (20)	1.5	0.8–2.7	0.20
≥2 prescriptions	16 (21)	33 (9)	3.0	1.5–6.0	0.002
Did not complete last prescription	9 (12)	14 (4)	3.5	1.4–8.3	0.004
On antimicrobial agent at admission	4 (5)	7 (2)	2.9	0.8–10.2	0.083
Time since antimicrobial agent‡					
No prior drug use	44 (59)	277 (75)	Referent		
≤3 mo	22 (29)	62 (17)	2.2	1.2–4.2	0.006
4–6 mo	9 (12)	30 (8)	1.7	0.8–4.5	0.12

*Odds ratio (OR) and 95% binomial confidence interval (95% CI). P value based on χ^2 test.
†Does not include antimicrobial agents that patient was taking at time of admission for bacteremic pneumococcal pneumonia.
‡For patients on >1 antimicrobial agent in previous 6 months, this represents time since most recent course of drugs.

increased odds of resistant infection, whereas patients exposed during the 4–6 months preceding infection did not have significantly increased odds of resistant infection. Finally, among patients who had at least 1 course of drugs, reporting that they did not finish the prescribed course was associated with >3-fold increased odds of a resistant infection compared to that for patients who reported completing the most recent course of antimicrobial agents (OR 3.5, 95% CI 1.4–8.3).

In multivariable analysis, prior exposure to macrolides (OR 2.8), prior influenza vaccination (OR 2.0), and Hispanic ethnicity (OR 4.1) were independently associated with an increased probability of macrolide resistance; a history of stroke (OR 0.2) was independently associated with a reduced probability of macrolide resistance (Table 3). All patients with a macrolide-resistant infection reported ≥1 of these 4 factors (prior exposure to macrolides, prior flu shot, Hispanic ethnicity, or no history of stroke). However, 97% of all study patients reported ≥1 of these 4 factors (data not shown). Among patients who reported at least 1 course of an antimicrobial agent in the 6 months preceding infection, the only significant characteristic of prior exposure was the patient's report that he or she failed to complete the full prescription (OR 3.4, 95% CI 1.2–9.9).

Discussion

In this case-control study of 444 adults with bacteremic pneumococcal pneumonia, we found that exposure to macrolides in the 6 months preceding infection, a history of influenza vaccination in the 12 months preceding infection, and Hispanic ethnicity were all independently associated with an increased probability of an erythromycin-resistant infection. However, most patients with erythromycin-resistant pneumococcal infections did not report any antimicrobial drug exposures in the 6 months preceding infection.

That prior antibacterial drug exposure is a risk factor for drug-resistant pneumococcal infections is supported by mathematical models and most empiric studies. Numerous studies have suggested a relatively strong association between prior antimicrobial drug use and the subsequent development of invasive infections due to penicillin-resistant pneumococcal infections (*19–24*). A recent case-control study comparing penicillin-susceptible to penicillin-nonsusceptible isolates from patients with pneumococcal bacteremia identified prior exposure to β-lactams, sulfonamides, and macrolides as risk factors; fluoroquinolone exposure was not a risk factor. These risk factors remained relevant up to 6 months before infection (*25*). Similarly, in another recent study of invasive pneumococcal disease comparing patients with macrolide-resistant isolates to macrolide-susceptible isolates, exposure to each of the following drugs was associated with an increased probability of a macrolide-resistant infection: penicillin, trimethoprim-sulfamethoxazole, clarithromycin, or azithromycin (*26*). While our current study found that exposure to the

Table 3. Independent risk factors for macrolide-resistant bacteremic pneumococcal pneumonia*

Risk factor	OR (95% CI)	p value
Macrolide ≤6 mo before infection	2.8 (1.4–5.8)	0.005
Influenza vaccination ≤1 mo before infection	2.0 (1.2–3.3)	0.013
Hispanic ethnicity	4.1 (1.4–12.5)	0.011
Prior stroke	0.2 (0.04–0.8)	0.021

*Odds ratio (OR), 95% confidence intervals (CI), and p value from logistic regression with all listed factors in the model.

macrolide drug class had the strongest association with the odds of a macrolide-resistant infection, the sample was too small to separately analyze the risk associated with different drugs within that class. However, given that antimicrobial drug exposure is common, research on modifiable risk factors for drug-resistant pneumococcal infections needs to focus on different patterns of exposure, both in terms of specific drugs selected and the dose and duration of administration. Among patients with a prior exposure to antimicrobial agents, reporting that they did not complete the course was significantly associated with the odds of a macrolide-resistant infection. Future studies correlating duration of therapy with risk for colonization with macrolide-resistant pneumococci would be useful to further explore this phenomenon.

Additional risk factors associated with drug-resistant pneumococcal infections have been reported to include extremes of age, attendance in daycare, having a household member in daycare, and coexisting illnesses, particularly HIV infection (*4*,*21*,*27*–*29*). However, many of these risk factors may be identified only because they are associated with higher probabilities of prior antimicrobial drug exposure, which may have been incompletely measured by our questions on prior drug use. In this study, for example, patients who report prior influenza vaccination may have increased access to health providers or increased frequency of respiratory infections, both factors that would likely increase the probability of prior antimicrobial drug exposure. Similarly, while we asked many questions about prior antimicrobial drug exposure, the observed association between erythromycin resistance and Hispanic ethnicity may be confounded by increased access to antimicrobial drugs through nontraditional sources (such as markets), where they may be less readily identified as antimicrobial agents (*30*). On the other hand, the identification of antimicrobial drug–independent risk factors would suggest that an additional mechanism, specifically increased exposure to persons with antimicrobial drug–resistant bacteria, is a factor promoting the emergence of macrolide-resistant pneumococcal infections. In this regard, the reduced probability of resistant infections seen in patients with a history of stroke might relate to relative social isolation in this population, which would reduce exposure to persons carrying drug-resistant pneumococci. Finally, some of the observed associations may be due to random (type I) error and represent false-positive results.

We did not enroll all patients with bacteremic pneumococcal pneumonia. Therefore, selection bias may have affected our assessment of different risk factors, particularly if enrollment differed for participants with macrolide-resistant and –macrolide-susceptible infections. Our analysis of the drug susceptibility of isolates from nonenrolled patients showed that the proportion of erythromycin-resistant isolates was not significantly different between enrolled and nonenrolled patients (data not shown). In addition, as pointed out by others, the selection of control groups affects the interpretation of results (*31*). In this study, we used patients with antimicrobial drug–susceptible pneumococcal infections as the control group to identify factors that might distinguish patients with pneumococcal infections at the time of treatment decisions. Finally, we relied primarily on patient self-report to identify prior antimicrobial drug exposures and the patterns of these exposures. We used a multistage, previously validated approach to measure exposure. Moreover, patient report is typically the source of information for providers at the time of treatment decisions. Although measurement error may have introduced bias in our risk estimates, the level of association between prior antimicrobial drug exposure and the odds of a macrolide-resistant infection were quantitatively similar when we used information from outpatient medical records.

More broadly, this study demonstrates that among patients with pneumococcal disease, patients with self-reported prior exposure to antimicrobial drugs, particularly macrolides, have an increased probability of infection with macrolide-resistant pneumococci. In addition, additional courses of antimicrobial drugs increase the probability of a drug-resistant infection. However, most patients with macrolide-resistant infections did not report any prior antimicrobial drug exposures. As a result, empiric therapy should be predominately guided by local susceptibility data rather than specific host characteristics.

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drug prescribing, drug resistance, and patient outcomes for community-acquired respiratory infections.

Appendix

The Delaware Valley Case Control Network includes the following physicians and laboratory directors listed with their respective hospitals.

Robert Dee, Herbert Auerbach (Abington Memorial Hospital); Jerry Zuckerman, Ierachmiel Daskal (Albert Einstein Medical Center); John Bartels, Stephen B. Chasko (Brandywine Hospital); Albert Keshgegian, Olarae Giger (Main Line Clinical Laboratory); Peter Spitzer, Bryn Mawr Hospital); Lawrence Livornese, (Lankenau Hospital); David Trevino (Paoli Hospital); Abby Huang, David Wright (Central Montgomery Medical Center); Dorothy Slavin, Mark Ingerman, Jerome Santoro, Lawrence Livornese, Ru Lin Ko Tung (Chestnut Hill Hospital); John Roberts, Jim Heald (Chester County Hospital); William Ravreby, Harvey B. Spector (Crozer Chester Medical Center, Taylor Hospital); Margaret Hessen (Springfield Hospital); Lawrence M. Matthews, Margaret Hessen (Delaware County Memorial Hospital); David Loughran, Rose M. Kenny (Doylestown Hospital); Donald Marcus, Xiaoli Chen (Elkins Park Hospital); Richard Tepper, Ila Mirchandani (Jeanes Hospital); Peter Axelrod (Fox Chase Cancer Center); Donald Marcus, Howard Elefant (Frankford Hospital Torresdale Division, Frankford Hospital Bucks County Campus, Frankford Hospital Frankford Division); Bonnie Rabinowitch, Fernando U. Garcia (Graduate Hospital); Abby Huang, Irwin Hollander (Grand View Hospital); Young S. Kim, Christopher Emery (Hahnemann University Hospital); Robert Dee, Pantaleon Fagel (Holy Redeemer Hospital and Medical Center); Paul Edelstein (Hospital of the University of Pennsylvania and Presbyterian Hospital); Paul McGovern (Presbyterian Hospital); Lorenzo M. Galindo (Mercy Fitzgerald, Mercy Suburban, Mercy Hospital of Philadelphia); William McNamee (Mercy Fitzgerald, Mercy Hospital of Philadelphia); Wayne Miller (Mercy Suburban Hospital); Robert Measley, Harvey J. Bellin (Methodist Hospital); David S. Fox, Paul Belser (Montgomery Hospital); Michael Braffman, John Stern, Gary Stopyra (Pennsylvania Hospital); Raymond Kovalski, Leonas Bekeris (Phoenixville Hospital); Raymond Kovalski, Dante DiMarzio (Pottstown Memorial Hospital); William McNamee, Susan Yaron (Riddle Memorial Hospital); Lawrence Livornese, Pradeep Bhagat (Roxborough Memorial Hospital); John Bartels, James Monihan (Jennersville Regional Medical Center); Robert Measley, John McCormick (St Agnes Medical Center); Abby Huang, David Steinberg, (St Luke's Quakertown); Donald Marcus, Zenon Gibas, Helen Kroh (St Mary Medical Center); Peter Axelrod, Allan Truant, Jamshid Moghaddas (Temple University Hospital, Northeastern Hospital, Episcopal Hospital); Jerry Zuckerman (Northeastern Hospital); Gregory Kane, Fred Gorestein, Donald Jungkind (Thomas Jefferson University Hospital); Donald Stieritz (Philadelphia VA Medical Center); David Loughran, Manjula Balasubramanian (Warminster Hospital).

References

1. Plouffe JF, Breiman RF, Facklam RR. Bacteremia with *Streptococcus pneumoniae*. Implications for therapy and prevention. Franklin County Pneumonia Study Group. JAMA. 1996;275:194–8.
2. Metlay JP. Antibacterial drug resistance: implications for the treatment of patients with community-acquired pneumonia. Infect Dis Clin North Am. 2004;18:777–90.
3. Whitney CG, Farley MM, Hadler J, Harrison LH, Lexau C, Reingold A, et al. Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States. N Engl J Med. 2000;343:1917–24.
4. Karlowsky JA, Thornsberry C, Jones ME, Evangelista AT, Critchley IA, Sahm DF. Factors associated with relative rates of antimicrobial resistance among *Streptococcus pneumoniae* in the United States: results from the TRUST Surveillance Program (1998–2002). Clin Infect Dis. 2003;36:963–70.
5. MacDougall C, Guglielmo BJ, Maselli J, Gonzales R. Antimicrobial drug prescribing for pneumonia in ambulatory care. Emerg Infect Dis. 2005;11:380–4.
6. Lonks JR, Garau J, Gomez L, Xercavins M, Ochoa de Echaguen A, Gareen IF, et al. Failure of macrolide antibiotic treatment in patients with bacteremia due to erythromycin-resistant *Streptococcus pneumoniae*. Clin Infect Dis. 2002;35:556–64.
7. Mandell LA, Bartlett JG, Dowell SF, File TM Jr, Musher DM, Whitney C. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. Clin Infect Dis. 2003;37:1405–33.
8. Mitchell AA, Cottler LB, Shapiro S. Effect of questionnaire design on recall of drug exposure in pregnancy. Am J Epidemiol. 1986;123:670–6.
9. Coulter A, Vessey M, McPherson K, Crossley B. The ability of women to recall their oral contraceptive histories. Contraception. 1986;33:127–37.
10. Beresford SA, Coker AL. Pictorially assisted recall of past hormone use in case-control studies. Am J Epidemiol. 1989;130:202–5.
11. Metlay JP, Hardy C, Strom BL. Agreement between patient self-report and a Veterans Affairs national pharmacy database for identifying recent exposures to antibiotics. Pharmacoepidemiol Drug Saf. 2003;12:9–15.
12. Koneman E, Allen S, Janda W, Schreckenberger P, Winn W Jr. Color atlas and textbook of diagnostic microbiology. 5th ed. Philadelphia: Lippincott-Raven; 1997.
13. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. 15th information supplement. Wayne (PA): The Committee; 2005.
14. Etest technical manual. Etest application sheet. *Streptococcus pneumoniae*. 2005 [cited 2006 May 29]. http://www.abbiodisk.com/pdf/etm_index.htm
15. Austrian R. The quellung reaction, a neglected microbiologic technique. Mt Sinai J Med. 1976;43:699–709.
16. Sorensen UB. Typing of pneumococci by using 12 pooled antisera. J Clin Microbiol. 1993;31:2097–100.
17. Pneumotest manual. Copenhagen: Statens Serum Institut; 2001.
18. Edelstein PH. Pneumococcal resistance to macrolides, lincosamides, ketolides, and streptogramin B agents: molecular mechanisms and resistance phenotypes. Clin Infect Dis. 2004;38(Suppl 4):S322–7.
19. Friedland IR. Comparison of the response to antimicrobial therapy of penicillin- resistant and penicillin-susceptible pneumococcal disease. Pediatr Infect Dis J. 1995;14:885–90.
20. Kronenberger CB, Hoffman R, Lezotte D, Marine W. Invasive penicillin-resistant pneumococcal infections: a prevalence and historical cohort study. Emerg Infect Dis. 1996;2:121–4.
21. Moreno S, Garcia-Leoni ME, Cercenado E, Diaz MD, Bernaldo de Quiros JC, Bouza E. Infections caused by erythromycin-resistant *Streptococcus pneumoniae*: incidence, risk factors, and response to therapy in a prospective study. Clin Infect Dis. 1995;20:1195–200.

22. Nava JM, Bella F, Garau J, Lite J, Morera MA, Marti C, et al. Predictive factors for invasive disease due to penicillin-resistant *Streptococcus pneumoniae*: a population-based study. Clin Infect Dis. 1994;19:884–90.
23. Pallares R, Gudiol F, Linares J, Ariza J, Rufi G, Murgui L, et al. Risk factors and response to antibiotic therapy in adults with bacteremic pneumonia caused by penicillin-resistant pneumococci. N Engl J Med. 1987;317:18–22.
24. Tan TQ, Mason EO Jr, Kaplan SL. Penicillin-resistant systemic pneumococcal infections in children: a retrospective case-control study. Pediatrics. 1993;92:761–7.
25. Ruhe JJ, Hasbun R. *Streptococcus pneumoniae* bacteremia: duration of previous antibiotic use and association with penicillin resistance. Clin Infect Dis. 2003;36:1132–8.
26. Vanderkooi OG, Low DE, Green K, Powis JE, McGeer A. Predicting antimicrobial resistance in invasive pneumococcal infections. Clin Infect Dis. 2005;40:1288–97.
27. Campbell GD Jr, Silberman R. Drug-resistant *Streptococcus pneumoniae*. Clin Infect Dis. 1998;26:1188–95.
28. Klugman KP. Pneumococcal resistance to antibiotics. Clin Microbiol Rev. 1990;3:171–96.
29. Reichler MR, Allphin AA, Breiman RF, Schreiber JR, Arnold JE, McDougal LK, et al. The spread of multiply resistant *Streptococcus pneumoniae* at a day care center in Ohio. J Infect Dis. 1992;166:1346–53.
30. Mainous AG III, Cheng AY, Garr RC, Tilley BC, Everett CJ, McKee MD. Nonprescribed antimicrobial drugs in Latino community, South Carolina. Emerg Infect Dis. 2005;11:883–8.
31. Harris AD, Karchmer TB, Carmeli Y, Samore MH. Methodological principles of case-control studies that analyzed risk factors for antibiotic resistance: a systematic review. Clin Infect Dis. 2001;32:1055–61.

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Antibody Response to *Pneumocystis jirovecii* Major Surface Glycoprotein

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We conducted a prospective pilot study of the serologic responses to overlapping recombinant fragments of the *Pneumocystis jirovecii* major surface glycoprotein (Msg) in HIV-infected patients with pneumonia due to *P. jirovecii* and other causes. Similar baseline geometric mean antibody levels to the fragments measured by an ELISA were found in both groups. Serum antibodies to MsgC in *P. jirovecii* patients rose to a peak level 3–4 weeks ($p<0.001$) after recovery from pneumocystosis; baseline CD4+ count ≥50 cells/μL and first episode of pneumocystosis were the principal host factors associated with this rise (both $p<0.001$). Thus, MsgC shows promise as a serologic reagent and should be tested further in clinical and epidemiologic studies.

Pneumocystis jirovecii, formerly known as *Pneumocystis carinii* special form *hominis* (*1*), is a leading cause of fatal pneumonia in HIV-positive persons and other immunosuppressed patients. Research on *P. jirovecii* has been hampered by the lack of a reliable in vitro culture system, so investigators have developed molecular techniques to characterize isolates. Studies by the Centers for Disease Control and Prevention, San Francisco General Hospital, and other medical centers in the United States that use these techniques have provided epidemiologic insights about *P. jirovecii* patients (*2*,*3*). Reports of *P. jirovecii* colonization detected by molecular probes in persons ranging from healthy young children to adults with chronic lung diseases raise the possibility that this organism may have medical and public health consequences beyond those on the immunocompromised host (*4*,*5*).

Serologic analysis may be useful in epidemiologic studies of *P. jirovecii* infection, especially in light of evidence that antibodies contribute to host defenses against the organism (*6–8*). Unfortunately, despite >40 years of investigation, a useful serologic test for *P. jirovecii* is not yet available (*9*,*10*). Antigens have mainly consisted of crude extracts from infected human or rodent lungs. These preparations have shown that antibodies to the organism are highly prevalent in the general population (*4*,*11*,*12*) but have been unable to distinguish present from past infection or colonization from active disease. Specific native *P. jirovecii* antigens have shown more promise as serologic reagents, but they are in short supply (*11*,*13*). This problem has been exacerbated by data about genetic diversity and host specificity of *Pneumocystis*, which have emphasized the importance of matching organisms used in studies with the host from which they have been derived (*1*).

More recent attention has turned toward the use of recombinant *P. jirovecii* antigens to study host immune responses (*14–19*). The major surface glycoprotein (Msg or gpA) is highly immunogenic and contains protective B- and T-cell epitopes, and the heavily glycosylated portion of the antigen plays a central role in the interaction of the organism with the host (*6–10*,*20*). Msg represents a family of proteins encoded by multiple genes and is thus capable of antigenic variation, which may serve as a mechanism to evade host immune responses. Our strategy has been to use a single Msg isoform that would enable us to begin to understand the host immune response to this complex glycoprotein. We developed 3 overlapping recombinant

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fragments (MsgA, MsgB, and MsgC), which span the entire length of the *P. jirovecii* Msg, and analyzed their reactivity with serum antibodies in different populations by Western blot (WB) and ELISA (*17*,*18*). A key finding in both studies was that asymptomatic, HIV-positive patients in Cincinnati with a past episode of *Pneumocystis* pneumonia (PCP) had a significantly higher degree of antibody reactivity to MsgC, the carboxyl terminus and most conserved part of the antigen, than patients who had never had the disease.

In this pilot study, we sought to determine whether serum antibody levels to MsgA, MsgB, and MsgC differed in HIV-positive patients with acute pneumonia due to *P. jirovecii* compared to those with pneumonia due to other causes. Further, we asked whether serum antibody levels would rise in these patients during treatment and recovery from pneumocystosis, which Msg fragment could best detect this increase, and whether specific host factors were associated with the antibody rise.

Materials and Methods

Patients and Study Design

As standard clinical practice, HIV-positive patients who came to San Francisco General Hospital with respiratory signs and symptoms compatible with pneumocystosis were evaluated by a uniform protocol that has been described previously (*21*). This protocol included obtaining specimens by induced sputum and, if necessary, bronchoscopy with bronchoalveolar lavage. Microscopic examination and cultures were used to establish a specific etiologic diagnosis. Consecutive patients undergoing sputum induction or bronchoscopy to diagnose PCP were enrolled in this study and provided written, informed consent to allow their medical records to be abstracted with a standardized data form. Study investigators classified patients as either PCP positive or PCP negative (controls), according to predetermined definitions that were blinded to serologic results. *Pneumocystis* patients were those patients with a microscopically confirmed diagnosis of *P. jirovecii*; these patients were treated with standard anti-*Pneumocystis* drugs as part of their regular medical care. Control patients were those whose microscopic examinations were negative for *P. jirovecii*, had *Pneumocystis* treatment discontinued, and recovered from acute pneumonia.

The study was conducted during a 4.5-year period (May 2000 through September 2004). During the first half of the study (2000–2002), an acute-phase serum specimen was drawn at the time of hospital admission for pneumonia, and a single convalescent-phase specimen was drawn at different intervals 5–12 weeks later. Preliminary analysis suggested that the *Pneumocystis* patients experienced a rise in mean serum antibody levels, whereas controls did not. Thus, during the later part of the study (2003–2004), additional serial convalescent-phase serum specimens were drawn every 1–2 weeks for 6 weeks from patients with pneumocystosis to measure early changes in antibody levels. Serum specimens were stored at −70°C and shipped to the University of Cincinnati for analysis. University of California San Francisco and University of Cincinnati institutional review boards approved the protocol.

Analysis of Serum Antibodies

Serum antibody levels to MsgA, MsgB, and MsgC were measured in a blinded manner by an ELISA as previously described (*14*,*17*,*18*). All serum specimens and the standard reference serum were diluted 1:100 and tested in duplicate wells of a 96-well plate against the following reagents: recombinant Msg fragments, *Escherichia coli* extract expressing the pET vector without insert (vector control), tetanus toxoid (TT) (positive control), and phosphate-buffered saline (PBS) without antigen (negative control). As an additional negative control, PBS was substituted for the serum specimen. Plates were washed, horseradish peroxidase (HRP)–labeled goat anti-human immunoglobulin G was added, plates were washed again, and tetramethylbenzidine substrate was added. The reaction was stopped by adding 0.18 mol/L H_2SO_4, and the plates were read at a wavelength of 450 nm. The reference serum specimen, which was obtained from a single person and had known reactivity to Msg, was run on each day as another control. HRP-labeled S-protein was used as a positive control and to correct for antigen loading. During the early part of the study, patient and reference serum specimens were tested at 1:100, 1:500 and 1:2,500 dilutions. The best results were obtained with the 1:100 dilution, so this dilution was used for the remainder of the study. The reactivity of each serum specimen to Msg was expressed as the ratio of reactivity to the pET vector: (mean optical density [OD] $Msg_{test\ serum}$ − mean OD $PBS_{test\ serum}$) / (mean OD $pET_{test\ serum}$ − mean OD $PBS_{test\ serum}$).

Statistical Analysis

Sex and racial distributions of *Pneumocystis* patients and controls were compared by using χ^2 tests of equality of proportions. Means and standard deviations were calculated to compare the center and spread of age and continuous parameters measured at enrollment (baseline). Mean values for *Pneumocystis* patients and controls were compared and tested for equality by using unpaired *t* tests with adjustment for unequal variances, either on the original or logarithmic scale. Geometric means and geometric standard deviations were calculated when data were approximately lognormally distributed, as judged by Shapiro-Wilks tests and visual assessment. Quantile-quantile plots of serum antibody levels showed that their distributions

were approximately lognormal, except for larger numbers of values at the lower quantiles. Pearson product moment correlations among clinical parameters and Msg fragments were obtained. Analyses of trends in mean values of each outcome were performed for all patients enrolled at baseline during either study period and followed up at least once at weeks 1–2, 3–4, or 5–6. Sparse data at later follow-up weeks precluded their inclusion in the regression analyses because of instability of parameter estimates. In addition, the numbers of patients observed at baseline and each follow-up time were too small to provide meaningful inferences from analysis.

Two stratifications of *Pneumocystis* patients were investigated with respect to trends and mean levels of Msg fragments: patients with CD4+ counts above and below the median value (≈50 cells/μL) and patients with and without a history of pneumocystosis. The analysis provided comparisons between means for patient categories at each follow-up week, as well as differences in patterns of change over time between patient groups. We obtained p values that compared mean values between periods and levels of CD4+ count or history of *Pneumocystis* pneumonia by using *t* tests with Tukey's adjustment for multiple comparisons. Patient-specific deviations from group means were included in the analysis as a random effect, which allowed the correlation between repeated measurements on the same patient over time to be included in between- and within-patient variance estimates. A p value <0.05 (2-tailed) was considered significant, unless stated otherwise. Analyses were performed by using the SAS procedure PROC MIXED (SAS for Windows version 9.2, SAS Institute Inc., Cary, NC, USA)

Results

Demographic and Clinical Characteristics

No significant differences were seen between the 80 *Pneumocystis* patients and the 41 control patients with other causes of pneumonia in terms of sex, race, or age (Table 1). Baseline serum albumin levels, arterial blood gas measurements, and the proportion of patients who required mechanical ventilation were also similar, which indicates that the overall general health of patients and the severity of pneumonia in these 2 groups were also comparable. In addition, the proportion of patients with prior pneumocystosis was similar in both groups. However, the *Pneumocystis* patients had more advanced or more poorly controlled HIV infection than did the controls. The *P. jirovecii* patients had a significantly lower geometric mean CD4+ count and a significantly higher mean plasma HIV RNA level than did control patients. Patients with pneumocystosis also exhibited a significantly higher mean serum lactate dehydrogenase (LDH) level than did controls; this finding is consistent with the observation that elevated serum LDH is a nonspecific indicator of PCP.

Table 1. Demographic characteristics and baseline clinical measurements of patients with *Pneumocystis* pneumonia (PCP) and controls*

Characteristic	PCP (n = 80)	Controls (n = 41)
Demographic		
Male (%)	86	76
Race (%)		
Caucasian	55	39
African American	29	39
Other	16	22
Age at enrollment (y)	41 ± 8	42 ± 8
Clinical		
CD4 count (cells/μL)†‡	29 ± 3	73 ± 4
Plasma HIV RNA (copies/mL)†‡	123,130 ± 5	15,582 ± 24
Albumin (g/L)	3.0 ± 0.5	3.1 ± 0.7
Prior PCP (%)	24	33
Pneumonia severity		
pO_2 (mm Hg)	65 ± 15.6	73 ± 29
Aa gradient (mm Hg)	44 ± 13	41 ± 15
LDH (U/L)†‡	359 ± 2	257 ± 2
Mechanical ventilation (%)	7	7
Serum antibody levels		
MsgA†	5.6 ± 4.8	3.7 ± 3.5
MsgB†	2.9 ± 1.3	1.9 ± 0.9
MsgC†	4.2 ± 4.3	4.3 ± 3.4

*Unless specified otherwise, values are mean ± standard deviation. pO_2, oxygen pressure; Aa, alveolar-arterial; LDH, lactate dehydrogenase; Msg, major surface glycoprotein.
†Geometric mean ± geometric standard deviation.
‡Significant difference between patient groups for CD4+ count ($p<0.01$), plasma HIV RNA ($p<0.001$), LDH ($p<0.001$).

Baseline and Sequential Serum Antibody Levels to MsgC

At the time of hospital admission for pneumonia, geometric mean serum antibody levels to MsgC in *Pneumocystis* patients and controls were similar (Table 1). Forty-one of 80 patients with PCP had ≥1 convalescent-phase serum specimen drawn in the first 6 weeks after hospital admission. The total number of patient visits was 62. Patients observed at weeks 3–4 (n = 19) had a higher mean serum antibody level to MsgC than the average level of all patients at baseline (n = 80) and patients observed at weeks 1–2 (n = 25) and weeks 5–6 (n = 18). Differences were significant, as determined by *t* statistics comparing group means, adjusted for paired comparisons ($p<0.01$ to $p<0.001$) (Table 2). Mean antibody levels at subsequent time points were 3.7 at 5–6 weeks, 4.0 at 7–8 weeks, and 3.0 at 9–12 weeks (data not shown).

Analysis of serum antibody levels in individual patients showed different patterns of reactivity (Figure). Eleven (58%) of the 19 patients studied at 3–4 weeks had an increase in their antibody levels, ranging from 1.4- to

Table 2. Antibody levels to major surface glycoprotein C in *Pneumocystis* pneumonia patients by CD4+ count and week of observation*

	All patients		CD4+ <50 cells/μL		CD4+ ≥50 cells/μL	
Week	n	Geometric mean (95% CI)	n	Geometric mean (95% CI)	n	Geometric mean (95% CI)
Baseline (0)	80	4.2 (3.1–5.8)	54	4.2 (2.8–6.3)	26	4.4 (2.5–7.6)
1–2	25	8.0 (4.1–15.8)	17	7.3 (3.0–17.8)	8	9.9 (2.8–35.3)
3–4	19	10.4 (4.7–23.1)†	13	6.3 (2.4–16.4)	6	30.4 (7.1–129.5)‡
5–6	18	3.7 (2.0–6.8)	10	5.5 (2.0–15.0)	8	2.3 (1.2–4.4)

*n = no. patients who were observed at the specified follow-up time and at baseline. CI, confidence interval.
†p<0.001 vs. week 0, p<0.001 vs. weeks 1–2, and p<0.001 vs. weeks 5–6.
‡p<0.001 vs. week 0, p<0.001 vs. weeks 5–6, p<0.03 vs. weeks 3–4 in CD4+ <50 cells/μL group.

22-fold above baseline levels. To determine if the rise in serum antibodies in the *Pneumocystis* patients at 3–4 weeks was specific for *P. jirovecii* or part of a broader increase in antibody reactivity, we examined the changes in antibody levels to TT in these 19 patients. The geometric mean antibody levels of 80 U at baseline and 117 U at 3–4 weeks were not significantly different.

Predictors of Serum Antibody Response to MsgC among *Pneumocystis* Patients

We examined clinical characteristics associated with an increase in antibody levels. No relationship was found between antibody level and sex, race, age, HIV viral load, serum albumin level, oxygenation, LDH level, or use of mechanical ventilation. CD4+ cell count (analyzed as CD4+ count <50 cells/μL or ≥50 cells/μL) was significantly associated with the ability to generate an increase in antibody levels (Table 2). Patients with CD4+ counts <50 cells/μL exhibited no significant changes in antibody levels to MsgC at any time point. In contrast, patients with CD4+ counts ≥50 cells/μL had a rise in mean antibody level, which peaked at 30.4 at 3–4 weeks and which was significantly higher than antibody levels at baseline (p<0.001) and at 5–6 weeks (p<0.001). The mean antibody level at 3–4 weeks in these patients was also significantly higher than the corresponding level in patients with <50 CD4+ cells (30.4 vs. 6.3, p<0.03).

The lack of a history of pneumocystosis also seemed to influence antibody response but not baseline antibody level. The mean antibody level to MsgC in the 59 patients with first episode of PCP was 4.1; this value was not significantly different from the mean value of 4.2 in the 19 patients with recurrent pneumocystosis (Table 3). Sequential antibody responses were compared in PCP patients whose episode of pneumonia was their first experience with the disease versus those whose pneumonia was a recurrent bout of the disease. Antibody levels in naive patients increased and reached a peak mean value of 17.9 at 3–4 weeks compared to 4.1 at baseline (p<0.001), whereas patients with a history of *P. jirovecii* pneumonia experienced no increase. Because the number of patients was low, we could not analyze the combined effects of PCP history and CD4+ cell count.

Baseline and Sequential Serum Antibody Levels to MsgA and MsgB

The baseline geometric mean serum antibody level to MsgA of 5.6 in PCP patients was not significantly different from the mean level of 3.7 in the controls (Table 1). Patients with pneumocystosis exhibited a different pattern of antibody response to MsgA than to MsgC in that the mean peak antibody levels to MsgA at 1–2 weeks and 3–4 weeks were similar (Table 4). However, none of the differences in antibody levels at different time points reached significance. In addition, no significant differences were found in the antibody levels at different time points in patients with and without a history of pneumocystosis or in patients with CD4+ cell counts <50 cells/μL or patients with CD4+ counts ≥50 cells/μL (data not shown).

No significant difference in baseline geometric mean serum antibody levels to MsgB was seen in patients with PCP and controls (Table 1). No significant differences were seen in antibody levels related to different time points, CD4+ counts, or history of pneumocystosis (data not shown).

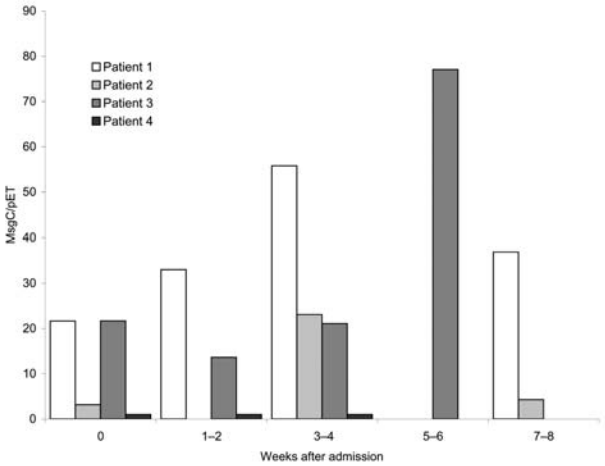

Figure. Sequential serum antibody responses to major surface glycoprotein C (MsgC) in HIV-positive patients with pneumocystosis. Patients 1 and 2: antibody levels rose from 21.6 and 3.2 at baseline (point 0) to 55.8 and 23.1 at 3–4 weeks and fell to 36.9 and 4.3, respectively, at 7–8 weeks; patient 3: antibody level showed few changes from baseline until it rose to 77.1 at 5–6 weeks; patient 4: antibody level remained at baseline level of 1.0 throughout.

Table 3. Antibody levels to major surface glycoprotein C in *Pneumocystis* pneumonia (PCP) patients by history of PCP and week of observation*

	No history of PCP		History of PCP	
Week	n	Geometric mean (95% CI)	n	Geometric mean (95% CI)
Baseline (0)	59	4.1 (2.8–6.0)	19	4.2 (2.0–9.0)
1–2	18	9.9 (4.6–21.5)	6	3.9 (0.5–30.8)
3–4	11	17.9 (5.9–53.6)†	6	3.0 (0.7–12.1)
5–6	13	4.4 (2.6–7.4)	4	1‡

*n = no. patients who were observed at the specified follow-up time and at baseline. CI, confidence interval.
†p<0.001 versus week 0.
‡Observed values were equal.

Discussion

Recombinant antigens derived from *P. jirovecii* have begun to attract attention as possible reagents for analyzing antibodies to *Pneumocystis* in humans (*14–19*). We have previously reported that HIV-positive, PCP-positive patients in Cincinnati had a significantly higher frequency and level of serum antibodies to MsgC than did HIV-positive, PCP-negative patients (*17,18*); this difference was not found with MsgA or MsgB. These patients were selected on the basis of a history of pneumocystosis and were clinically healthy. The present pilot study has extended these observations to HIV-positive patients hospitalized with acute pneumonia due to *P. jirovecii* and other causes (controls) in San Francisco. The goal of the first part of this study was to determine if baseline antibody levels to the 3 Msg fragments in PCP patients differed from those in the controls. Data showed that the geometric mean antibody levels to MsgC, MsgA, and MsgB were similar in both groups.

In the second part of this study, we analyzed the sequential changes in antibody levels to the 3 Msg fragments in *P. jirovecii* patients after treatment and recovery from pneumocystosis. The results showed a significant rise in mean antibody levels to MsgC that reached a peak at 3–4 weeks. In contrast to MsgC results, no significant changes in antibody levels to MsgA or MsgB occurred at any time point. The pattern of antibody reactivity to these Msg fragments also differed to some degree from the pattern of reactivity to MsgC. Taken together, these data suggest that MsgC is the best Msg fragment to use to analyze antibody responses in this population of HIV-positive patients with active *Pneumocystis* pneumonia.

An antibody rise by 3–4 weeks occurred in 58% of the *Pneumocystis* patients we studied. Of the potential host factors that could affect antibody responses, we were most interested in CD4+ cells, HIV RNA level, and previous history of pneumocystosis. Patients with CD4+ cell counts <50 cells/μL did not mount an antibody response, whereas patients with CD4+ counts ≥50 cells/μL that peaked 3–4 weeks after diagnosis showed a vigorous antibody response. In contrast to CD4+ count, mean viral load in *P. jirovecii* patients was not associated with an increase in antibody levels. Previous reports have shown that CD4+ cells and HIV itself affect antibody responses in HIV-positive patients, which can be reversed by highly active antiretroviral therapy (HAART) (*22–24*). In HIV-positive patients who are severely immunocompromised and have experienced opportunistic infections such as pneumocystosis, however, this immune reconstitution may be incomplete (*25*). Perhaps some who did not respond to treatment fall into this category, but we did not have information about HAART use and immune reconstitution in this cohort.

Our data showed that pneumocystosis patients with or without a previous episode of the disease had similar baseline antibody levels to MsgC; however, patients who experienced their first bout of PCP had better antibody responses after recovery from the disease. Those who were experiencing a recurrent bout of pneumocystosis may have been unable to mount an antibody response to previous episodes and remained at risk. CD4+ cell count or differences in the treatment of pneumocystosis might also play a role in the ability of patients without a previous history of pneumocystosis to mount an antibody response, but our analysis was not powered to analyze multiple factors simultaneously.

Comparison of our results with previous work is complicated by the fact that these earlier studies were performed in the pre-HAART era and involved crude or native antigens. Analysis of whether a detectable antibody

Table 4. Antibody levels to major surface glycoprotein A in *Pneumocystis* pneumonia (PCP) patients by history of PCP and week of observation*

	Combined		No history of PCP		History of PCP	
Week	n	Geometric mean (95% CI)	n	Geometric mean (95% CI)	n	Geometric mean (95% CI)
Baseline (0)	78	5.6 (3.9–7.9)	59	5.8 (3.9–8.7)	19	4.7 (2.1–10.5)
1–2	24	12.0 (6.7–21.4)	18	13.7 (7.0–26.8)	6	7.6 (1.2–47.4)
3–4	17	11.5 (5.2–25.3)	11	13.7 (4.3–43.5)	6	7.3 (1.1–48.9)
5–6	17	5.1 (2.3–11.3)	13	4.0 (1.9–8.2)	4	5.7 (0.2–207.9)

*n = no. patients who were observed at the specified follow-up time and at baseline. CI, confidence interval.

response to *Pneumocystis* antigens could develop in HIV-positive patients who recovered from pneumocystosis produced conflicting results (*9–11,26–30*). One report showed a rise in antibodies to native Msg in 43% of HIV-positive patients; host factors such as CD4+ count or pO_2 could not distinguish responders from nonresponders (*13*). A more recent study that used different recombinant Msg constructs than we used found that HIV-negative, immunocompromised patients who recovered from pneumocystosis had increased antibody levels, but HIV-positive patients who recovered had lower levels and poor antibody responses (*16*). One factor that may contribute to these disparate results is antigenic variation, which involves differences in the Msg constructs themselves. We have developed several variants of our current Msg construct and found that they differ in their ability to distinguish among HIV-positive patients who have and not have had pneumocystosis (unpub. data). PCP patients exhibit greater reactivity with multiple MsgC clones than do patients without PCP or blood donors, but whether the antigens that are recognized are cross-reactive or clone specific is unclear. Further studies to identify broadly reactive MsgC antigens associated with recovery from PCP, as well as proteins (e.g., Kex1) encoded by single-copy genes, would be of interest (*19*).

The sequential serologic results reported here, which were obtained from a limited number of patients, provide the basis for a large, prospective, multisite study of sequential antibody responses to MsgC in HIV-positive patients who have pneumonia caused by *P. jirovecii* and other organisms. Serologic surveys need to be performed in different areas to determine which Msg fragment is the predominant fragment recognized by HIV-positive patients and healthy persons. Standardizing Msg antigen preparations, ELISA conditions, and data analysis would be helpful so that serologic results could be reproduced in different laboratories.

The development of a successful serologic test for *Pneumocystis* infection will have clinical and epidemiologic applications. Serologic tests with MsgC might be used in the diagnosis of pneumonia in situations (e.g., in developing countries) in which a specific cause cannot be established; in cohort studies to investigate the relationship of serum antibody levels and the risk for, and recovery from, *Pneumocystis* pneumonia; in seroepidemiologic surveys and outbreaks of pneumocystosis; and in investigating the pathogenic role for *P. jirovecii* in chronic lung diseases in which colonization of the organism has been detected (*5*).

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References

1. Redhead SA, Cushion MT, Frenkel JK, Stringer JR. *Pneumocystis* and *Trypanosoma cruzi*: nomenclature and typifications. J Eukaryot Microbiol. 2006;53:2–11.
2. Beard CB, Carter JL, Keely SP, Huang L, Pieniazek NJ, Moura IN, et al. Genetic variation in *Pneumocystis carinii* isolates from different geographic regions: implications for transmission. Emerg Infect Dis. 2000;6:265–72.
3. Crothers K, Beard CB, Turner J, Groner G, Fox M, Morris A, et al. Severity and outcome of HIV-associated *Pneumocystis* pneumonia containing *Pneumocystis jirovecii* dihydropteroate synthase gene mutations. AIDS. 2005;19:801–5.
4. Vargas SL, Hughes WT, Santolaya ME. Search for primary infection by *Pneumocystis carinii* in a cohort of normal, healthy infants. Clin Infect Dis. 2001;32:855–61.
5. Morris A, Sciurba FC, Githaiga A, Lebedeva I, Elliott WM, Hogg JC, et al. Association of chronic obstructive pulmonary disease severity and *Pneumocystis* colonization. Am J Respir Crit Care Med. 2004;170:408–13.
6. Gigliotti F, Hughes WT. Passive immunoprophylaxis with specific monoclonal antibody confers partial protection against *Pneumocystis carinii* pneumonitis in animal models. J Clin Invest. 1988;81:1666–8.
7. Garvy BA, Wiley JA, Gigliotti F, Harmsen AG. Protection against *Pneumocystis carinii* pneumonia by antibodies generated from either T helper 1 or T helper 2 responses. Infect Immun. 1997;65:5052–6.
8. Zheng M, Shellito JE, Marrero L, Zhong Q, Julian S, Ye P, et al. CD4+ T cell–independent vaccination against *Pneumocystis carinii* in mice. J Clin Invest. 2001;108:1469–74.
9. Smulian AG, Walzer PD. Serological studies of *Pneumocystis carinii* infection. In: Walzer PD, editor. *Pneumocystis carinii* pneumonia. New York: Marcel Dekker; 1994. p. 141–51.
10. Walzer PD. Immunological features of *Pneumocystis* infection. In: Cushion MT, Walzer PD. *Pneumocystis* pneumonia. New York: Marcel Dekker; 2005. p. 451–78.
11. Peglow SL, Smulian GA, Linke MJ, Crisler J, Phair JWM, Gold J, et al. Serologic responses to specific *Pneumocystis carinii* antigens in health and disease. J Infect Dis. 1990;161:296–306.
12. Smulian AG, Sullivan D, Linke MJ, Halsey N, Quinn T, MacPhail AP, et al. Geographic variation in the humoral response to *Pneumocystis carinii*. J Infect Dis. 1993;167:1243–7.
13. Lundgren B, Lundgren JD, Nielsen T, Mathiesen L, Nielsen JO, Kovacs JA. Antibody responses to a major *Pneumocystis carinii* antigen in human immunodeficiency virus–infected patients with and without *P. carinii* pneumonia. J Infect Dis. 1992;165:1151–5.
14. Garbe TR, Stringer JR. Molecular characterization of clustered variants of genes encoding major surface antigens of human *Pneumocystis carinii*. Infect Immun. 1994;62:3092–101.
15. Mei Q, Turner RE, Sorial V, Klivington D, Angus CW, Kovacs JA. Characterization of major surface glycoprotein genes of human *Pneumocystis carinii* and high-level expression of a conserved region. Infect Immun. 1998;66:4268–73.
16. Bishop LR, Kovacs JA. Quantitation of anti-*Pneumocystis jiroveci* antibodies in healthy persons and immunocompromised patient. J Infect Dis. 2003;187:1844–8.

17. Daly KR, Fichtenbaum C, Tanaka R, Linke MJ, Obert R, Thullen T, et al. Serologic responses to epitopes of the major surface glycoprotein of *Pneumocystis jiroveci* differ in human immunodeficiency virus–infected and uninfected persons. J Infect Dis. 2002;186:644–51.
18. Daly KR, Koch J, Levin L, Walzer PD. Enzyme-linked immunosorbent assay and serologic responses to *Pneumocystis jiroveci*. Emerg Infect Dis. 2004;10:848–54.
19. Kutty G, Kovacs JA. A single-copy gene encodes Kex1, a serine endoprotease of *Pneumocystis jiroveci*. Infect Immun. 2003;71:571–4.
20. Theus SA, Andrews RP, Stelle P, Walzer PD. Adoptive transfer of lymphocytes sensitized to the major surface glycoprotein of *Pneumocystis carinii* confers protection in the rat. J Clin Invest. 1995;95:2587–93.
21. Huang L, Stansell JD. AIDS and the lung. Med Clin North Am. 1996;80:775-801.
22. Malaspina A, Moir S, Orsega SM, Vasquez J, Miller NJ, Donoghue ET, et al. Compromised B cell responses to influenza vaccination in HIV-infected individuals. J Infect Dis. 2005;191:1442-50.
23. De Milito A. B lymphocyte dysfunctions in HIV infection. Curr HIV Res. 2004;2:11-21.
24. Jacobson MA, Khyam-Bashi H, Martin JN, Black D, Ng V. Effect of long-term highly active antiretroviral therapy in restoring HIV-induced abnormal B-lymphocyte function. J Acquir Immune Defic Syndr. 2002;31:472–7.
25. D'Amico R, Yang Y, Mildvan D, Evans SR, Schnizlein-Bick CT, Hafner R, et al. Lower CD4+ T lymphocyte nadirs may indicate limited immune reconstitution in HIV-1 infected individuals on potent antiretroviral therapy: analysis of immunophenotypic marker results of AACTG 5067. J Clin Immunol. 2005;25:106–15.
26. Burns SM, Read JA, Yap PL, Brettle RP. Reduced concentrations of IgG antibodies to *Pneumocystis carinii* in HIV-infected patients during active *Pneumocystis carinii* infection and the possibility of passive immunization. J Infect. 1990;20:33–9.
27. Hofmann B, Odum N, Cerstoft J, Platz P, Ryder LP, Svjgaard A, et al. Humoral response to *Pneumocystis carinii* in patients with acquired immunodeficiency syndrome and in immunocompromised homosexual men. J Infect Dis. 1985;152:838–40.
28. Hofmann B, Nielsen PB, Odum N, Gerstoft J, Platz P, Ryder LP, et al. Humoral and cellular responses to *Pneumocystis carinii*, CMV, and herpes simplex in patients with AIDS and in controls. Scand J Infect Dis. 1988;20:389–94.
29. Elvin K, Bjorkman A, Heurlin N, Eriksson BM, Barkholt L, Linder E. Seroreactivity to *Pneumocystis carinii* in patients with AIDS verus other immunosuppressed patients. Scand J Infect Dis. 1994;26:33–40.
30. Laursen AL, Andersen PL. Low levels of IgG antibodies against *Pneumocystis carinii* among HIV-infected patients. Scand J Infect Dis. 1998;30:495–9.

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Virulent Epidemics and Scope of Healthcare Workers' Duty of Care

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The phrase "duty of care" is, at best, too vague and, at worst, ethically dangerous. The nature and scope of the duty need to be determined, and conflicting duties must be recognized and acknowledged. Duty of care is neither fixed nor absolute but heavily dependent on context. The normal risk level of the working environment, the healthcare worker's specialty, the likely harm and benefits of treatment, and the competing obligations deriving from the worker's multiple roles will all influence the limits of the duty of care. As experts anticipate the arrival of an avian influenza pandemic in humans, discussion of this matter is urgently needed.

Epidemiologists are warning against an impending pandemic of avian influenza that could kill several million people (*1*). This possibility raises an urgent and thorny ethical question: Are healthcare professionals obligated to care for patients during virulent epidemics of infectious disease?

Duty of Care

Duty of care, in the medical context, is often invoked as a sort of quasi-biblical commandment, akin to "do not lie" or "do not murder." In a document submitted to the Severe Acute Respiratory Syndrome (SARS) Expert Panel Secretariat, Godkin and Markwell suggest that policy guidelines on the duty of care (which they term duty *to* care) should state that healthcare professionals' duty to care extends to a public health emergency in outbreak conditions (*2*). The authors however suggest that healthcare employers have a set of reciprocal responsibilities toward their staffs, which include duties to inform, protect, and support healthcare personnel. Singer et al., in an article on the ethical issues raised by SARS in Toronto, briefly discuss the duty to care before concluding that the 9 authors "could not reach consensus on the issue of duty of care, particularly regarding the extent to which healthcare workers are obligated to risk their lives in delivering clinical care" (*3*). The term "duty of care" (which I take to be synonymous with duty to care) is, at best, too vague and, at worst, ethically dangerous. For these reasons, the phrase should be modified in favor of more specific descriptions of the obligations of healthcare workers.

Special Obligation of Doctors to Benefit Their Patients

By virtue of their profession, doctors and nurses have more stringent obligations of beneficence than most. They have obligations to a specified group of persons (their patients) that nonmedical personnel have no obligation to help. The term "duty of care" refers to these special obligations. In its bare form, however, the phrase gives no indication of the precise nature of the duty, nor of its limits. Its definitional vagueness, combined with its rhetorical appeal, may be used to justify actions without the need for rational deliberation. During the SARS outbreaks in Toronto, the phrase was often used as a self-standing argument for active involvement on the part of medical staff, without any critical examination of its meaning. Used in this manner, the term may become a subtle instrument of intimidation, pressuring healthcare workers into working in circumstances that they consider morally, psychologically, or physically unacceptable. The phrase duty of care can thus be ethically dangerous by giving the illusion of legitimate moral justification.

To be of any use, the phrase needs to be fleshed out. Are there limits to the duty? Should doctors do everything in their power to benefit their patients? The answer, surely, is no. Doctors are under no moral obligation to donate one of their kidneys to one of their patients, for example. They may, of course, choose to do so, but their act would exceed the demands of everyday morality. What distinguishes normal duty from acting beyond the call of duty, however, is not always clear-cut; the boundary between the 2 categories is fuzzy (*4*).

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Contingency of the Limits of Duty of Care

Defining the limits of the duty of care is a daunting task, strewn with philosophical and logistical difficulties. As the example of the kidney-giving doctors shows, the duty is not absolute but, rather, constrained by several factors. First, the limits of the duty should be a function of the normal risk level. A doctor practicing in Kinshasa, Democratic Republic of Congo (DRC), for instance, is going to incur more risk than a doctor in rural Dorset, England. The diseases are many and the facilities few in DRC. Every nurse or doctor, by accepting a post, is usually aware of the perils of treating infected patients. The appearance of an exotic, highly virulent disease, however, challenges healthcare workers to question their interpretation of the duty of care, in particular, its limits. This challenge was apparent both in the HIV/AIDS epidemics of the 1980s in the United States and in the 2003 SARS outbreaks in Toronto, in which doctors and nurses refused to treat afflicted patients on the grounds that they presented too great a danger (*2*,*5*). This phenomenon is also likely to occur if the anticipated avian influenza epidemic affects Western hospitals. In light of these historical precedents, hospitals may want to inform prospective staff members of what is expected in crisis situations before, rather than in the midst of, an emergency. By using comparisons and statistics, hospitals could indicate the sorts of risks healthcare staff are expected to handle.

Another factor in defining acceptable risk levels relates to the healthcare worker's specialty. Within the same hospital, an emergency care physician, as a first responder to many critically ill or injured persons, is obviously more at risk than, for example, a dermatologist. By entering into a specialty, doctors implicitly consent to a range of risks and responsibilities associated with the job. The outer limit of acceptable personal risk will fall further along the continuum of risk for some specialists (e.g., infectious disease physicians) than for others (e.g., dermatologists or rheumatologists). During the SARS outbreaks in Toronto, the persons most at risk were nurses and infectious diseases (ID) specialists. As a result of their specialist training, they may have felt a stronger obligation to participate than doctors in other areas of medicine.

Doctors as Multiple Agents

Doctors, although they belong to their own professional community and adhere to its set of rules, are also part of the broader community and therefore subject to the same rights and duties as other members. The 2 spheres of obligation, professional and personal, are both separate and overlapping. They are separate in that the obligations of doctors toward their patients give them rights that nonmedical members of the society do not possess, such as opening someone's abdomen to remove an appendix. The spheres are overlapping, however, in that their role as doctors does not completely absolve their responsibilities as members of the broader community. The immunity from sanction is specific, not general. A gynecologist may legitimately examine intimate parts of his or her patient but cannot drive beyond the speed limit or steal apples from the market stall. With the acquisition of additional duties and rights conferred by the profession, the doctor also agrees to relinquish certain rights enjoyed by others. By entering into the profession, a doctor agrees not only to abide by new rules but also to accept dangers that would be unacceptable to many (e.g., performing a delicate, invasive procedure on a patient with hepatitis or HIV/AIDS).

In times of crisis, the duties deriving from doctors' multiple roles may come into conflict. Doctors, for instance, may have a duty to care for their SARS or avian influenza–infected patients as well as a duty to care for their own children by protecting them (and hence themselves) from infection. So a further problem with the duty to care, aside from its vagueness, is that it fails to consider the holder of the duty as a multiple agent belonging to a broader community. Doctors, in such situations, play several incompatible roles—doctor, spouse, parent, for example—and they must deal with them as best they can. The limits of the duty of care are thus also defined by the strengths of competing rights and duties.

Virtues of Patients and Their Duty of Care

Whereas much has been written on what makes a good doctor, scant attention has been devoted to the good patient (*6*,*7*). Pellegrino and Thomasma, in For the Patient's Good, devote a chapter to the "good patient" (*8*). "Patients," they write, "must relate to physicians in all of the virtuous ways that govern human interrelationships and social conduct" (*8*). The authors identify 4 key virtues for the good patient: truthfulness, compliance, tolerance, and trust. The virtue most pertinent to this discussion is tolerance. In their examination of tolerance, Pellegrino and Thomasma mention the patients' need to understand the limitations and fallibility of medicine and to care for the well-being of their fellow patients (*8*).

The virtue of tolerance should also require patients to acknowledge healthcare workers' plurality of roles, as well as their fears and concerns in the face of severe risk. If these fears are well founded and reach such a level that medical staff are worried for their life or that of their loved ones, the virtuous patient ought to allow them to step down from their role as caregivers. In such cases, insisting that they continue in this role would reflect a lack of compassion and understanding. Patients should be entitled to ask for a replacement who is less anxious or prone to panic, but they cannot force other persons to undergo extreme stress against their wishes.

When a physician visited the 1995 Ebola virus outbreak in Kikwit (DRC), he found 30 dying patients in an abandoned hospital, left to care for themselves amid rotting corpses, sometimes in the same bed (*9*). Was the last doctor justified in leaving the patients, or should he or she have been obliged to single-handedly treat the highly and dangerously infectious Ebola patients? The answer depends, at least in part, on the actual risk to the doctor and the potential benefits (including the alleviation of pain and distress) that his or her presence will bring to the patients. If the actual risk for serious illness or death for the doctor is low and the benefits of treatment substantial, then he or she may have an obligation to remain. If, however, the lack of protective equipment means that the chances of infection are high and no, or trivially small, benefits will result for the patients (as is often the case with Ebola), then the doctor may justifiably abandon the doomed patients. Virtuous patients, aware of the high risk and the futility of treatment, would not force a doctor to care for them in such circumstances. Patients too have a duty to care for healthcare workers. Part of this duty is not to require doctors to transcend the bounds of reasonable risk during treatment and to respect and acknowledge their roles outside the realm of medicine.

As potential participants in the drama and as holders of a duty of care toward healthcare workers, the general public also should be involved in setting limits to duty. Some form of dialogue between the public and the medical profession, through the media, public consultations, and educational establishments, could help establish a mutually acceptable set of limits.

Impact on Patient Trust

The justified abandonment of patients by doctors arguably will result in the harm or even death of these patients. Moreover, public trust in doctors will diminish as persons realize that they, like the 30 forsaken Ebola patients at Kikwit General Hospital, might be left on their own as soon as the risk reaches a certain level. The patients at Kikwit died alone, abandoned by both medical staff and their own frightened relatives. So tragic is the situation that it seems counterintuitive to justify the actions of the nurses and doctors. Yet, before passing judgment, comparing this situation with another hypothetical situation may be useful.

If a swimmer in an isolated but supervised beach starts to drown 50 meters from the shore, the lifeguard may reasonably be expected to attempt a rescue. This, after all, is the lifeguard's duty as a qualified professional. If, however, the person is drowning 2 miles out and is surrounded by a school of hungry, man-eating sharks, then one cannot expect the solitary lifeguard to dive among the sharks to save the swimmer, even if that means the swimmer will certainly die and even if the lifeguard has a small chance of saving him or her (at great personal risk).

The lifeguard cannot be criticized for not interfering, even though his or her prima facie duty is to rescue drowning persons. Likewise, the fact that doctors can, in exceptional circumstances, refuse to treat patients does not necessarily entail a moral wrong, no matter how serious the consequences to the abandoned patients. As long as patients hold realistic expectations of the limits of doctors' duty of care, no trust should be lost when these limits are transgressed.

Urgent Need

In the last 20 years, various outbreaks of severe infectious diseases, from Ebola virus infection to SARS, have highlighted the need for a more precise account of the duties and obligations of healthcare professionals. The impending avian influenza epidemic makes such an account urgent. The concept of duty of care, in its bare form, is too vague to be helpful. Its limits are not fixed, but contingent on various factors, from the working environment's normal risk level to the healthcare worker's specialty and the range of other obligations that derive from his or her multiple roles. To clarify this overlooked topic, empirical social science research should be conducted to illuminate the views and reasoning of physicians, patients, and members of the public on the limits of the duty of care. Philosophical reflection on the issue as well would do much to clarify this overlooked topic. As dramatic as it may sound, delineating the limits of the duty of care may prevent large numbers of doctors from abandoning their patients in a crisis. Such abandonment has happened in the past and may occur again.

In light of the potentially catastrophic impact of avian influenza on human health and economic well-being, this topic should engender a burst of activity and debate in hospitals, universities, and medical journals. We should explore not only the nebulous limits of the duty of care but also infection control measures, staff training and involvement, the role of medical students and volunteers, the triaging of incoming patients, and the logistics of treatment, depending on the severity of the epidemic, as well as the lessons learned from past epidemics. However difficult the task, these issues should best be tackled now, in times of relative calm, rather than in times of pandemic turbulence.

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References

1. British Broadcasting Company News Online. Bird flu "could kill 150m people." 2005 Sep 15 [cited 2006 Jun 5]. Available from http://news.bbc.co.uk/2/hi/asia-pacific/4292426.stm
2. Godkin D, Markwell H. The duty to care of healthcare professionals: ethical issues and guidelines for policy development. Toronto: Joint Center for Bioethics, University of Toronto; 2003.
3. Singer P, Benatar S, Bernstein M, Daar AS, Dickens BM, MacRae SK, et al. Ethics and SARS: lessons from Toronto. BMJ. 2003;327:1342–4.
4. Heyd D. Supererogation: its status in ethical theory. Cambridge (UK): Cambridge University Press; 2002.
5. Zuger A, Miles S. Physicians, AIDS, and occupational risk. JAMA. 1987;258:1924–8.
6. Sokol D. How (not) to be a good patient. J Med Ethics. 2004;6:612.
7. Campbell A, Swift T. What does it mean to be a virtuous patient? Virtue from the patient's perspective. Scottish Journal of Healthcare Chaplaincy. 2002;5:29–35.
8. Pellegrino E, Thomasma D. For the patient's good. New York: Oxford University Press; 1988.
9. Virus. British Broadcasting Company Radio. 1999 Mar 3.

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Human and Canine Pulmonary Blastomycosis, North Carolina, 2001–2002

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We investigated a cluster of blastomycosis in 8 humans and 4 dogs in a rural North Carolina community. Delayed diagnosis, difficulty isolating *Blastomyces dermatitidis* in nature, and lack of a sensitive and specific test to assess exposure make outbreaks of this disease difficult to study.

Blastomyces dermatitidis is the etiologic agent of blastomycosis, a fungal disease that affects humans and animals, particularly dogs. The ecologic niche for *B. dermatitidis* is not fully understood, although research suggests acquisition of blastomycosis may be associated with environmental conditions such as sandy soil, organic matter, waterways, and earth-disturbing activities (*1–4*). In North America, the southeastern and south central states and parts of the Midwest, Canada, and New York have been identified as areas where the disease is endemic (*1*). Most sporadic and outbreak cases have also been reported from these regions (*4*). In states where blastomycosis is reportable, the annual incidence is 1.3–1.4 cases per 100,000 persons. However, areas of hyperendemicity can have rates of up to 41.9 cases per 100,000 persons (*5*). Furthermore, incidence of this disease has been increasing in certain regions (*6*). This report describes a recent cluster of human and canine pulmonary blastomycosis that occurred in rural North Carolina.

The Study

From November 2001 to February 2002, pulmonary blastomycosis was diagnosed in 8 residents of a small town in Duplin County, located in eastern North Carolina. In contrast, 1 human case of pulmonary blastomycosis was identified in Duplin County from January 1995 through June 2001. Four patients attended the same school. Three cases of canine blastomycosis with onset in late December 2001 and January 2002 were also diagnosed by a veterinarian in the same town; canine blastomycosis had not been diagnosed for at least 7 years before 2002.

Active case finding was conducted from September 2001 to February 2002. Duplin County death certificate and hospital discharge information was reviewed for blastomycosis. Clinical laboratories and infection control programs for major hospitals in eastern North Carolina were queried for blastomycosis or unusual pneumonia cases. Workers at high occupational risk for blastomycosis (e.g., construction crews, cemetery workers, and county road scrapers) were also contacted. All county veterinarians were contacted to identify additional canine cases.

To explore commonalities among cases, surviving patients or family members of dead patients were interviewed by using a standardized questionnaire. Information was obtained on patient demographics, recreational and work-related activities, medical history, and pet ownership. Information was limited to the hospital record for 1 patient. Because half of the patients attended the same school, an environmental assessment of the school grounds was performed. Finally, historical climatic data were obtained to compare conditions during and before the outbreak. Demographic and clinical data for human cases are summarized in Table 1.

The epidemic plotting of this outbreak suggests ongoing exposure (Figure), as was the experience in previous North Carolina blastomycosis outbreaks (*7,8*). However, neither the epidemiologic investigation nor the environmental assessment showed a common source for human and canine exposure. The only commonality among the students was involvement with different outdoor afterschool activities. The remaining patients did not frequent the school grounds. Two of the adult patients lived within 0.4 km of each other. Otherwise, no commonalities were noted in hobbies, occupation, or recreational activities between patients. None of the canine cases had contact with the patients or other infected dogs. One dog was primarily an indoor dog; another was kept in an outside run for 3 months before illness caused by suspected rabies exposure. Symptom onset was first recognized in humans, although the epidemic curve suggests that human and canine patients were exposed during the same period.

Several environmental conditions, such as soil type and pH, proximity to waterways, elevation of nearest waterway, temperature, precipitation, and earth-disturbing activities might facilitate growth of *B. dermatitidis* and have been associated with blastomycosis (*1–4*,*9*). Duplin County is located in the middle coastal plain region of the

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Table 1. Demographic and clinical features of human blastomycosis patients, Duplin County, North Carolina, 2001

Feature	Value
Median age, y (range)	25 (15–82)
Race, no. (%)	
African American	5 (63)
White	3 (37)
Sex, no. (%)	
Male	6 (75)
Clinical signs and symptoms, no. (%)	
Cough	5 (71)
Fever	6 (86)
Chest pain	6 (86)
Shortness of breath	4 (57)
Clinical outcomes, no. (%)	
Hospitalized	8 (100)
Pneumonia diagnosed	8 (100)
Failed antimicrobial drug treatment	8 (100)
Treated with itraconazole*	6 (75)
Survived	7 (87)

*One patient with multiple underlying medical problems was treated with amphotericin B; another patient was treated with fluconazole.

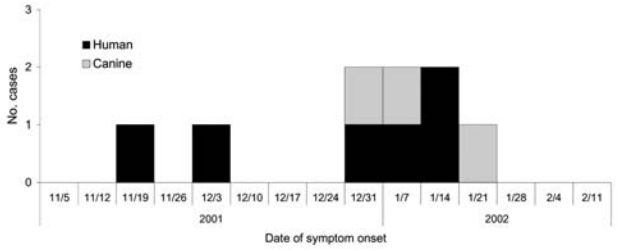

Figure. Epidemic curve of a human and canine pulmonary blastomycosis outbreak, North Carolina, 2001–2002.

state, 34 m above sea level, and is characterized by acidic soil and multiple soil types. No major lakes or rivers are located in the area of interest. Environmental conditions at the home sites for patients are shown in Table 2.

The mean county temperatures were 13.8°C for October through December 2001 and 12.1°C for these 3 months from 1996 to 2000. Mean total precipitation was 8.6 cm for October through December 2001 compared with 21.3 cm for these 3 months for 1996 to 2000.

During the fall of 2001, two construction projects were taking place at the school, and crops were harvested in a newly cultivated field near the school. However, nonstudent patients had no known contact with the school grounds. Furthermore, no one with high occupational risk for exposure had symptoms consistent with blastomycosis during the time of interest.

Conclusions

This investigation underscores the difficulty in identifying the source for a blastomycosis outbreak. Little success has been achieved in isolating *B. dermatitidis* from soil, especially without a potential common exposure site (*10*). Because the median incubation period for blastomycosis is 30–45 days (*11*) the environmental conditions at the exposure site often differ between the exposure period and the time of the outbreak investigation. No investigations, including 2 other investigations in North Carolina, have achieved cultural confirmation of an environmental source in the absence of good epidemiologic evidence (*7*,*8*). For this reason, environmental testing was not performed in this investigation. Sources suggest that canine blastomycosis might predate human cases (*12*), but human cases were identified first in this cluster.

Although isolating *B. dermatitidis* from the environment is challenging, certain conditions have been associated with blastomycosis in earlier outbreaks and may have contributed to this one. For example, the acidic pastureland of the area and proximity of patient homes to low-lying waterways are both consistent with sites of other outbreaks (*2*,*4*,*9*). Although excavations have been implicated in previous outbreak investigations of blastomycosis (*1*,*3*), the earth-disturbing activities in this outbreak could not account for nonstudent patients. Although most patient home sites were located on soils containing sand, none were pure sand. Humidity and precipitation may encourage release of *B. dermatitidis* spores (*1*,*3*). In North Carolina, the average relative humidity does not vary greatly from season to season but is generally highest in winter (*13*). Precipitation during the months in question was diminished compared with the previous 5 years, and only 3 days of rain were recorded in November 2001.

Diagnostic testing for blastomycosis can be problematic. Because of poor sensitivity and specificity, the skin test antigen blastomycin is not available, and serologic tests also show poor specificity (*14*). The diagnostic standard is

Table 2. Environmental conditions at human blastomycosis patients' homes, Duplin County, North Carolina, 2001*

Patient	Proximity to nearest major stream, m	Proximity to nearest minor stream, m	Elevation of nearest major stream, m	Elevation of nearest minor stream, m	Soil type
1	4,575.21	498.07	25.48	36.65	Norfolk loamy sand
2	3,047.30	398.11	25.03	32.98	Marvin and Gritney
3	6,602.32	1,073.54	39.36	36.04	Woodington loamy fine sand
4	4,105.22	591.10	44.84	40.82	Rains fine sandy loam
5	3,541.88	767.95	44.84	39.65	Rains fine sandy loam
6	4,773.45	395.70	44.84	39.13	Goldsboro loamy sand

*Home site address was unavailable for 2 case-patients.

visualization of the yeast form of *B. dermatitidis* in a clinical specimen (*11*). Culture is ideal, but the organism may take up to 5 weeks to grow (*15*). Furthermore, obtaining a positive specimen may require invasive techniques. Finally, a user-friendly rapid test to determine population exposure is not available for blastomycosis.

This report illustrates the challenges to investigating blastomycosis clusters. Research suggests that rapid growth of *B. dermatitidis* may be promoted by local environmental conditions, which may have been the case in the present outbreak. However, outbreak investigations of this rare but potentially serious condition would be more conclusive with the ability to isolate the organism from the environment, timely diagnosis of the disease, and availability of a sensitive and specific screening test to assess population exposure.

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References

1. Baumgardner DJ, Burdick JS. An outbreak of human and canine blastomycosis. Rev Infect Dis. 1991;13:898–905.
2. Baumgardner DJ, Steber D, Glazier R, Paretsky DP, Egan G, Baumgardner AM, et al. Geographic information system analysis of blastomycosis in northern Wisconsin, USA: waterways and soils. Med Mycol. 2005;43:117–25.
3. Proctor ME, Klein BS, Jones JM, Davis JP. Cluster of pulmonary blastomycosis in a rural community: evidence for multiple high-risk environmental foci following a sustained period of diminished precipitation. Mycopathologia. 2002;153:113–20.
4. Klein BS, Vergeront JM, DiSalvo AF, Kaufman L, Davis JP. Two outbreaks of blastomycosis along rivers in Wisconsin. Isolation of *Blastomyces dermatitidis* from riverbank soil and evidence of its transmission along waterways. Am Rev Respir Dis. 1987;136:1333–8.
5. Centers for Diseases Control and Prevention. Blastomycosis acquired occupationally during prairie dog relocation—Colorado, 1998. MMWR Morb Mortal Wkly Rep. 1999;48:98–100.
6. Dworkin MS, Duckro AN, Proia L, Semel JD, Huhn G. The epidemiology of blastomycosis in Illinois and factors associated with death. Clin Infect Dis. 2005;41:e107–e11.
7. Centers for Disease Control and Prevention. Blastomycosis—North Carolina. MMWR Morb Mortal Wkly Rep. 1976;25:205–6.
8. Smith JG Jr, Harris JS, Conant NF, Smith DT. An epidemic of North American blastomycosis. JAMA. 1955;158:641–6.
9. Dwight PJ, Naus M, Sarsfield P, Limerick B. An outbreak of human blastomycosis: the epidemiology of blastomycosis in the Kenora catchment region of Ontario, Canada. Can Commun Dis Rep. 2000;26:82–91.
10. Klein BS, Vergeront JM, Weeks RJ, Kumar UN, Mathai G, Varkey B, et al. Isolation of *Blastomyces dermatitidis* in soil associated with a large outbreak of blastomycosis in Wisconsin. N Engl J Med. 1986;314:529–34.
11. Chapman S. *Blastomyces dermatitidis*. In: Mandell G, Bennett J, Dolin R, editors. Principles and practice of infectious diseases. Philadelphia: Churchill Livingstone; 2000. p. 2733–46.
12. Sarosi GA, Eckman MR, Davies SF, Laskey WK. Canine blastomycosis as a harbinger of human disease. Ann Intern Med. 1979;91:733–5.
13. State Climate Office of North Carolina. General summary of North Carolina climate. 2006 [cited 2006 May 15]. Available from http://www.nc-climate.ncsu.edu/climate/ncclimate.html
14. Bradsher RW, Chapman SW, Pappas PG. Blastomycosis. Infect Dis Clin North Am. 2003;17:21–40.
15. Martynowicz MA, Prakash UBS. Pulmonary blastomycosis: an appraisal of diagnostic techniques. Chest. 2002;121:768–73.

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West Nile Virus Epizootiology, Central Red River Valley, North Dakota and Minnesota, 2002–2005

Jeffrey A. Bell,* Christina M. Brewer,* Nathan J. Mickelson,* Gabriel W. Garman,* and Jefferson A. Vaughan*

West Nile virus (WNV) epizootiology was monitored from 2002 through 2005 in the area surrounding Grand Forks, North Dakota. Mosquitoes were tested for infection, and birds were surveyed for antibodies. In 2003, WNV was epidemic; in 2004, cool temperatures precluded WNV amplification; and in 2005, immunity in passerines decreased, but did not preclude, WNV amplification.

West Nile virus (WNV) is a flavivirus with an enzootic cycle that involves primarily mosquitoes and birds in the order Passeriformes. Since its introduction into the northern prairies of the United States in 2002, WNV has flourished. In 2003 and 2005, the prairie states of North Dakota, South Dakota, and Nebraska recorded the highest incidence of cases in humans (per 100,000 county residents) for the entire United States (*1*). Although WNV is still new to the region, the ecology of the northern prairie seems to offer favorable conditions for its continued enzootic transmission. This report chronicles the initial establishment of WNV within the central Red River Valley of eastern North Dakota and northwestern Minnesota (Figure).

The Study

Host-seeking mosquitoes were collected in and around Grand Forks, North Dakota, by using Mosquito Magnet traps (American Biophysics Corp., North Kingston, RI, USA) for 4 transmission seasons, from early summer 2002 through fall 2005. Mosquitoes were sorted by species and tested for WNV by using reverse transcriptase PCR assays. WNV was detected only in *Culex tarsalis* (*2*).

Passerine birds in and around Grand Forks were surveyed for antibodies to WNV for 3 transmission seasons: June 24–October 27, 2003, April 4–July 7, 2004, and May 17–August 11, 2005. In 2003 and early 2004, birds were captured by using mist nets, blood (≤0.1 mL) was obtained by brachial venipuncture, and birds were released. Later in 2004 and in 2005, necropsies were performed on dead birds. Blood spots were placed on filter paper and later eluted in 250 μL saline. Samples were tested for anti-WNV antibodies by using a qualitative epitope-blocking ELISA (*3*).

This is the first report of seroprevalence of WNV in passerines in the northern prairies. A total of 277 birds (11 species) were tested (Table 1). In 2003, seroprevalence was relatively low (17%). The first seropositive bird was captured July 24, 2003, 4 days after the first WNV-positive pool of *Cx. tarsalis* was detected (*2*). Most seropositive birds (11 of 14) were collected in September, when migratory species were leaving and vector populations were waning. Thus, a lag occurred between peak abundance of infected vectors in mid- to late August 2003 (*2*) and seroconversion of passerines. Seroprevalence rates were significantly higher in 2004 and 2005 than in 2003 (Table 1, Fisher exact tests, $p<0.0001$) and were higher than most seroprevalences reported for passerines in the eastern and southeastern regions of the United States (*4–6*). All passerine species sampled in 2004 and 2005 contained seropositive birds, which indicated that all these species were preyed on by vectors regardless of differences in their nesting habitats (e.g., cattail marshes, peridomestic). American robins, common grackles, and red-winged blackbirds showed increased seroprevalence from 2003 to 2004. High seroprevalence was maintained in passerines in 2005 despite low WNV activity (i.e., low natural boosting) during 2004 (*2*), which suggests that passerine immunity to WNV may last longer than a single season (*7*,*8*).

Surprisingly, American crows had a high seroprevalence to WNV. Previous laboratory and field studies have indicated that most American crows die so quickly from WNV infection that they never have time to seroconvert (*9–11*). Why crows in the Red River Valley survive WNV infection is not known. One possibility is that WNV has undergone genetic changes with a concurrent loss in virulence as it spread westward from forest ecosystems with

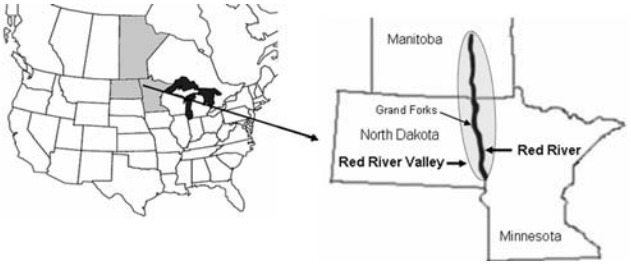

Figure. Red River Valley of North Dakota, Minnesota, and Manitoba.

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Table 1. Prevalence of antibodies against West Nile virus (WNV) in 11 species of passerine birds sampled within the central Red River Valley of North Dakota and Minnesota during 2003, 2004, and 2005

Common name	Scientific name	% birds with antibodies to WNV (n)		
		2003	2004	2005
American crow	*Corvus brachyrhynchos*	–	33 (6)	50 (6)
American robin	*Turdis migratorius*	18 (17)	50 (6)	38 (26)
Brown-headed cowbird	*Molothrus ater*	–	–	17 (6)
Blue jay	*Cyanocitta cristata*	50 (4)	–	87 (8)
Brewer's blackbird	*Euphagus cyanocephalus*	–	–	33 (3)
Common grackle	*Quiscalus quiscula*	0 (11)	71 (14)	63 (67)
Eastern kingbird	*Tyrannus tyrannus*	–	–	100 (3)
European starling	*Sturnus vulgaris*	–	100 (2)	67 (3)
Gray catbird	*Dumetella carolinensis*	–	75 (4)	–
House sparrow	*Passer domesticus*	20 (45)	–	50 (2)
Red-winged blackbird	*Agelaius phoeniceus*	0 (5)	50 (20)	63 (19)
Total		17.1 (82)	57.7 (52)	57.3 (143)

Cx. pipiens and *Cx. restuans* as its primary vectors into prairie ecosystems with *Cx. tarsalis* as its primary vector (*2*,*12*,*13*).

Annual data on seroprevalence in passerines, environmental temperatures, reporting of human cases, and minimum infection rates (MIR) in vector populations are summarized in Table 2.

Conclusions

Environmental conditions from 2002 to 2005 produced a natural field experiment, which demonstrated the differing magnitudes by which environmental temperature and host immunity affected local WNV activity. Despite warm temperatures and high vector abundance, WNV activity was low during its introductory year (2002), as indicated by low numbers of human cases, undetectably low seasonal MIR, and by low seroprevalence in passerines in 2003. Because WNV had only recently arrived, presumably there was neither sufficient time nor number of infection nidi to promote extensive amplification cycles. However, 2003 was an epidemic year for WNV, as indicated by the increased number of human cases statewide and the high MIR in the local vector population. The relatively low level of immunity in passerines at the time also likely contributed to the epidemic. In 2004, unusually cool environmental temperatures prolonged vector larval development, adult emergence, and the arboviral extrinsic incubation period. As a result, duration of the 2004 transmission season was nearly half that of the preceding seasons (Table 2). Thus, WNV activity during 2004 was low (i.e., reduced vector abundance, number of human cases, and seasonal MIR), and the virus had insufficient time to undergo extensive amplification cycles, similar to the situation that occurred during the introductory year of 2002.

However, the epidemic conditions of 2003 had produced a high level of herd immunity in the local bird population in 2004. This immunity carried over into 2005. (Note: most migratory passerine species live for several years and return each year to the same general locale to breed.) In 2005, environmental temperature, length of transmission season, and vector abundance were all nearly identical to those of the epidemic year of 2003. Yet the intensity of WNV activity during 2005 was considerably less than that during 2003. The big difference between

Table 2. Epizootiology of West Nile virus (WNV) within the central Red River Valley of North Dakota and Minnesota during the first 4 years of its introduction into the region*

Year	Primary transmission season†	Thermal accumulations (degree-days)‡	Vector abundance§	Human cases in ND¶	Seasonal MIR#	Passerine seroprevalence
2002, introductory	92 days (11 Jun–10 Sep)	1,067	230	17	0.0 (n = 5,871)	No birds tested
2003, epidemic	92 days (11 Jun–10 Sep)	1,022	21	617	5.7 (n = 5,432)	17% (n = 82)
2004, cold	51 days (7 Jul–1 Sep)	371	9	20	0.0 (n = 1,245)	58% (n = 52)
2005, equilibrium?	84 days (20 Jun–11 Sep)	867	29	86	1.3 (n = 3,123)	57% (n = 143)

*ND, North Dakota; MIR, minimum infection rate.
†Time between first and last appearances of host-seeking *Culex tarsalis* mosquitoes in Mosquito Magnet traps.
‡Based on developmental threshold temperature of 14.3°C for WNV growth in *Cx. tarsalis* (*14*).
§Average number of *Cx. tarsalis* mosquitoes captured per trap-night in Grand Forks, ND.
¶Data from North Dakota Department of Public Health (*15*).
#No. of WNV-infected *Cx. tarsalis* mosquitoes per 1,000.

2003 and 2005 was the level of immunity in passerines. The high prevalence of immunity during 2005 may have contributed to preventing another epidemic, but it did not totally eliminate WNV activity. Levels of WNV activity during 2005 (as measured by human cases and mosquito MIR) were intermediate between those of the epidemic year (2003) and both the introductory year (2002) and the coldest year (2004). Thus, environmental temperature dictated the ultimate success (or more precisely, the failure) of WNV amplification within the central Red River Valley during 2004, whereas even high levels of herd immunity among the reservoir population exerted only a moderating effect on WNV activity during 2005.

Whether the level of WNV activity observed during 2005 will be representative of the arbovirus's natural equilibrium within the central Red River Valley remains to be seen. One uncertainty is how seasonal transmission is initiated. Is WNV reintroduced every spring through infected migratory birds or wind-blown mosquitoes from the south? Or does WNV survive the harsh winters inside mosquitoes undergoing diapause, only to reemerge in the spring? Either or both of these scenarios could be correct, but one conclusion is certain: the incidence of WNV disease in horses from North Dakota during June of 2002 and again in May of 2005 (*15*) indicates that WNV becomes active in the northern Great Plains well in advance of the first summer brood of its primary vector, *Cx. tarsalis* (*2*).

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References

1. Centers for Disease Control and Prevention. West Nile virus—statistics, surveillance and control, January 10, 2006 [cited 2006 Jan 30]. Available from http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm.
2. Bell JA, Mickelson NJ, Vaughan JA. West Nile virus in host-seeking mosquitoes within a residential neighborhood in Grand Forks, North Dakota. Vector Borne Zoonotic Dis. 2005;5:373–82.
3. Blitvich BJ, Marlenee NL, Hall RA, Calisher CH, Bowen RA, Roehrig JT, et al. Epitope-blocking enzyme-linked immunosorbent assays for the detection of serum antibodies to West Nile virus in multiple avian species. J Clin Microbiol. 2003;41:1041–7.
4. Komar N, Burns J, Dean C, Panella NA, Dusza S, Cherry B. Serological evidence for West Nile virus infection in birds in Staten Island, New York, after an outbreak in 2000. Vector Borne Zoonotic Dis. 2001;1:191–8.
5. Godsey MS, Blackmore MS, Panella NA, Burkhalter K, Gottfried K, Halsey LA, et al. West Nile virus epizootiology in the Southeastern United States, 2001. Vector Borne Zoonotic Dis. 2005;5:82–8.
6. Beveroth TA, Ward MP, Lampman RL, Ringia AM, Novak RJ. Changes in seroprevalence of West Nile virus across Illinois in free-ranging birds from 2001 through 2004. Am J Trop Med Hyg. 2006;74:174–9.
7. Howard JJ, Oliver J, Grayson MA. Antibody response of wild birds to natural infection with alphaviruses. J Med Entomol. 2004;41:1090–103.
8. Gibbs SEJ, Hoffman DM, Stark LM, Marlenee NL, Blitvich BJ, Beaty BJ, et al. Persistence of antibodies to West Nile virus in naturally infected rock pigeons (*Columba livia*). Clin Diagn Lab Immunol. 2005;12:665–7.
9. Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, et al. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis. 2003;9:311–22.
10. Yaremych SA, Warner RE, Mankin PC, Brawn JD, Raim A, Novak R. West Nile virus and high death rate in American crows. Emerg Infect Dis. 2004;10:709–11.
11. Caffrey C, Smith SCR, Weston TJ. West Nile virus devastates an American crow population. Condor. 2005;107:128–32.
12. Davis CT, Beasley DWC, Guzman H, Pushker R, D'Anton M, Novak RJ, et al. Genetic variation among temporally and geographically distinct West Nile virus isolates, United States, 2001, 2002. Emerg Infect Dis. 2003;9:1423–9.
13. Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD. Genetic and phenotypic variation of West Nile virus in New York, 2000–2003. Am J Trop Med Hyg. 2004;71:493–500.
14. Reisen WK, Fang Y, Martinez VM. Effects of temperature on the transmission of West Nile virus by *Culex tarsalis* (Diptera:Culicidae). J Med Entomol. 2006;43:309–17.
15. North Dakota Department of Public Health. North Dakota West Nile virus surveillance program, January 3, 2006 [cited 2006 Jan 30]. Available from http://www.ndwnv.com

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O'nyong-nyong Virus, Chad

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We report the first laboratory-confirmed human infection with O'nyong-nyong virus in Chad. This virus was isolated from peripheral blood mononuclear cells of a patient with evidence of a seroconversion to a virus related to Chikungunya virus. Genome sequence was partly determined, and phylogenetic studies were conducted.

On November 2, 2004, a febrile 19-year-old French soldier staying in Chad and returning from a mission in Sarh, in the southern part of the country, was admitted to the hospital. Clinical examination showed a high body temperature (38°C), rash, periocular erythema, and pharyngitis. Abdominal, cardiopulmonary, and neurologic functions were normal. Except for body temperature, biochemical and hematologic values were normal. Serologic results were negative for *Rickettsia typhi*, *R. conorii*, *Legionella pneumophila*, *Bordetella pertussis*, HIV, and human herpesviruses 1 and 2. Results of malaria testing were also negative. The patient received intravenous acetaminophen for 2 days, according to the protocol used by the French Armed Forces Medical Service in the event of fever occurring overseas. He recovered after 5 days without sequelae.

Serum samples, collected during the acute phase (November 3–5, 2004) and after (November 23 and December 7, 2004; January 10 and February 1, 2005) were transported to our laboratory and tested by ELISA for immunoglobulin M (IgM) and IgG antibodies to a battery of arboviruses by IgM-antibody capture (MAC-ELISA) and antigen-capture ELISA, respectively (*1*). Each serum sample was considered positive if the optical density (OD) ratio, OD (viral antigen)/OD (uninfected cells), was >3. The first sample (November 3, 2004) contained no antibodies (OD ratio <2) to dengue viruses, West Nile virus, Wesselsbron virus, Rift Valley fever virus, Bunyamwera virus, or Chikungunya virus (CHIKV). Remaining samples contained antibodies to a virus serologically related to CHIKV (OD ratios >3) for both IgM (sample 2 and following samples) and IgG (sample 3 and those following). Antibody titers peaked 20 days (IgM) and 68 days (IgG) after the onset of symptoms (Figure 1). The IgM titer returned to a low level within 2 months after onset of illness.

Results of CHIKV-specific real-time reverse transcription–PCRs (*2*) performed with serum samples as templates were negative. Virus isolation was attempted by incubation of peripheral blood mononuclear cells collected on the day of onset with C6/36 (*Aedes albopictus*) and Vero (E6 clone) monolayers. After 5 days, supernatants were collected and used to infect fresh cell cultures. After 2 days, cytopathologic effects were observed in Vero monolayers; a high level of cell death was also observed in C6/36 cells. Infected Vero and C6/36 cells were then examined by indirect immunofluorescence assay (IFA) for 8 different alphaviruses with alphavirus-specific antibodies and in-house mouse hyperimmune ascitic fluids to CHIKV, Mayaro (MAYV), Tonate (TONV), Semliki Forest (SFV), and Sindbis (SINV) viruses. Results of IFA were positive when alphavirus-specific antibodies and antibodies to CHIKV, MAYV, and TONV were used; no fluorescence was observed when antibodies to SFV and SINV were used at the dilution 1:200.

CHIKV-specific real-time RT-PCRs were negative when cell culture supernatants were used as samples; this result excluded CHIKV as the etiologic agent. We next used cM3W and M2W2 alphavirus-specific primers (*3*) to partially amplify viral genome by RT-PCR. The RT-PCR product was sequenced (GenBank accession no. DQ381540, isolate IMTSSA/5163) and used in a BLAST search that identified O'nyong-nyong virus (ONNV, E value $6e^{-130}$).

Viral RNA was amplified for phylogenetic studies by using ONNV-specific primers for nsP3, E2, and E1 sequences (primer sequences are available on request). RT-PCR products (614, 728, and 1080-nt long, respectively) were sequenced (GenBank accession nos. DQ383272, DQ383273, and DQ399055, respectively) and compared with ONNV sequences available on GenBank database;

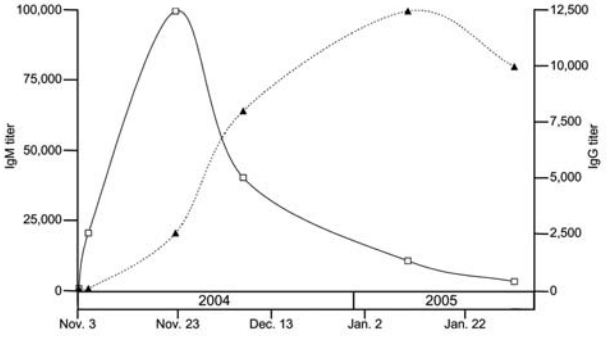

Figure 1. Immunoglobulin M (IgM) (□) and IgG (▲) titer in serum. For all samples, antibody titers were determined by serial dilution assays. Antibody titer is defined as the reciprocal of the highest dilution of serum that yields a positive serologic reaction.

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alignments were performed with ClustalW 1.7 software. Comparison of partial sequences showed a high degree of homology between the virus we isolated in Chad and strains previously isolated. In all 4 regions sequenced, paired identity at the nucleotide and amino acid level ranged from 92% to 98% and from 95% to 98%, respectively. Compared with ONNV isolate Gulu, the nsP3 sequence of our isolate featured a glycine-encoding codon deletion (nt 5249–5251, according to ONNV strain Gulu numbering). This deletion was also observed in ONNV strains SG650 and IbH10964, which might indicate a common lineage.

Four phylograms were constructed, each based on 1 genomic region we sequenced. Among ONNV sequences, all 4 phylograms exhibited a similar pattern: ONNV isolate IbH 10964 (Nigeria) and ONNV strain SG650 (Uganda) seemed to be closely related, whereas isolates Gulu (Uganda) and IMTSSA/5163 (Chad) were placed in 2 different branches (100% bootstrap value in the 4 phylograms). The phylogenetic tree based on E1-encoding sequence (1080-nt long) gave the opportunity to include 2 other ONNV sequences (Figure 2).

ONNV (family *Togaviridae*, genus *Alphavirus*) was first isolated from human blood and anopheline mosquitoes in Gulu, Uganda, in 1959 (*4*) and has been responsible for several outbreaks in humans that occurred in East Africa (Kenya, Uganda, Tanzania, Malawi, Mozambique). Fever, headache, joint pains, and rash were the principal signs and symptoms (*5*,*6*). Virus isolations from human and animal sera as well as from *Anopheles funestus* and *A. gambiae* have been reported in East Africa (*7*,*8*). Human and animal infections based on serologic evidence have also been reported in Nigeria, Ghana, and Sierra Leone (*9*,*10*). ONNV was also isolated from sentinel mice in Senegal and caused an outbreak in Côte d'Ivoire in the 1980s (*11*). To our knowledge, ONNV had never before been isolated in Chad.

The Sarh region, where the patient was infected, consists predominantly of plains covered with a mixture of grasses and woodlands. This region receives heavy rainfall during the 6-month rainy season, from May to October. To our knowledge, no recent data are available concerning the presence of *Anopheles* spp. in this region. Patient infection occurred outside any reported outbreak involving ONNV or another arbovirus. Moreover, the mission involved 9 other French soldiers whose serum specimens, collected a few weeks after their return from Sarh and transported to our laboratory, did not show serologic evidence of infection with an alphavirus.

SFV antibodies failed to detect ONNV by IFA, although both viruses are members of the same antigenic complex (*12*). The distribution of ONNV strains observed

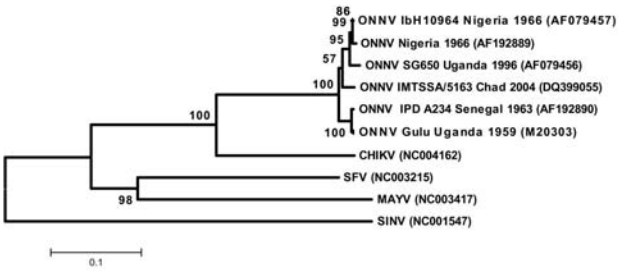

Figure 2. Phylogenetic tree of O'nyong-nyong virus (ONNV) based on partial E1 nucleotide sequence. Phylogram was constructed with MEGA 2 program (http://megasoftware.net/mega2.html) and tree drawing used the Juke-Cantor algorithm for genetic distance deter of divergence). CHIKV (Chikungunya virus), SFV (Semliki Forest virus), MAYV (Mayaro virus), and SINV (Sindbis virus) sequences have been introduced for correct rooting of the tree.

in the phylograms seemed to be independent of viral isolation locations or years. This finding suggests either a high level of viral genomic sequence stability over time or the circulation of ONNV strains across Africa, which has given rise to a mixing of ONNV strains from different origins in the same areas. However, because of the limited number of sequences available for genetic comparison, this observation on the distribution of ONNV strains needs confirmation.

In the absence of virus isolation, the diagnosis of infections with ONNV is difficult because of the close antigenic relationship of this virus with other alphaviruses, especially CHIKV. Development of a specific serologic assay for ONNV within the SFV antigenic complex would be a valuable tool for diagnosis and surveillance studies.

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Dr Bessaud spent 5 years as a research assistant in the tropical virology unit at the Tropical Medicine Institute of the French Armed Forces Medical Service. He is involved in the diagnosis of outbreaks due to arboviruses; he also researches the flavivirus-encoded protease complex.

References

1. Peyrefitte CN, Pastorino BA, Bessaud M, Gravier P, Tock F, Couissinier-Paris P, et al. Dengue type 3 virus, Saint Martin, 2003–2004. Emerg Infect Dis. 2005;11:757–61.

2. Pastorino B, Bessaud M, Grandadam M, Murri S, Tolou HJ, Peyrefitte CN. Development of a TaqMan RT-PCR assay without RNA extraction step for the detection and quantification of African Chikungunya viruses. J Virol Methods. 2005;124:65–71.
3. Pfeffer M, Proebster B, Kinney RM, Kaaden OR. Genus-specific detection of alphaviruses by a semi-nested reverse transcription-polymerase chain reaction. Am J Trop Med Hyg. 1997;57:709–18.
4. Haddow AJ, Davies CW, Walker DH. O'nyong-nyong fever: an epidemic virus disease in East Africa. Trans R Soc Trop Med Hyg. 1960;54:517–22.
5. Marshall TF, Keenlyside RA, Johnson BK, Chanas AC, Smith DH. The epidemiology of O'nyong-nyong in the Kano Plain, Kenya. Ann Trop Med Parasitol. 1982;76:153–8.
6. Sanders EJ, Rwaguma EB, Kawamata J, Kiwanuka N, Lutwama JJ, Ssengooba FP, et al. O'nyong-nyong fever in south-central Uganda, 1996–1997: description of the epidemic and results of a household-based seroprevalence survey. J Infect Dis. 1999;180:1436–43.
7. Williams MC, Woodall JP, Corbet PS, Gillett JD. O'nyong-nyong fever: an epidemic virus disease in East Africa. 8. Virus isolations from *Anopheles* mosquitoes. Trans R Soc Trop Med Hyg. 1965;59:300–6.
8. Williams MC, Woodall JP, Gillett JD. O'nyong-nyong fever: an epidemic virus disease in East Africa. VII. Virus isolations from man and serological studies up to July 1961. Trans R Soc Trop Med Hyg. 1965;59:186–97.
9. Guyer B. Serological survey for arboviruses in Igbo-Ora, western Nigeria. Ann Trop Med Parasitol. 1972;66:243–50.
10. Woodruff AW, Bowen ET, Platt GS. Viral infections in travellers from tropical Africa. BMJ. 1978;1:956–8.
11. Lhuillier M, Cunin P, Mazzariol MJ, Monteny N, Cordellier R, Bouchite B. Epidémie rurale à virus "Igbo Ora" (avec transmission inter-humaine) en Côte d' Ivoire en 1984–1985. Bull Soc Pathol Exot. 1988;81:386–95. PMID: 2846194
12. Calisher CH, Shope RE, Brandt W, Casals J, Karabatsos N, Murphy FA, et al. Proposed antigenic classification of registered arboviruses I. Togaviridae, Alphavirus. Intervirology. 1980;14:229–32.

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O'nyong-nyong virus
[o-nyong′nyong]

O'nyong-nyong means "severe joint pain" in the language of the Acholi people of East Africa. O'nyong-nyong virus was first isolated in Uganda in 1959 at the beginning of an outbreak that spread to Kenya, Tanzania, Zaire, Malawi, and Mozambique. One of the largest arbovirus epidemics ever recorded, the outbreak lasted until 1962 and affected >2 million persons. A species of the genus *Alphavirus* and closely related to chikungunya virus, O'nyong-nyong virus is transmitted by the bite of anopheline mosquitoes and causes an acute, self-limited, febrile illness characterized by lymphadenitis and joint pain.

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003 and wikipedia.org

Human Bocavirus in French Children

Vincent Foulongne,* Yann Olejnik,* Virginie Perez,* Stéphane Elaerts,* Michel Rodière,* and Michel Segondy*

Human bocavirus (HBoV), a new member of the genus *Bocavirus* in the family *Parvoviridae*, has been recently associated with respiratory tract infections. We report the epidemiologic and clinical features observed from a 1-year retrospective study of HBoV infection in young children hospitalized with a respiratory tract infection.

Viral respiratory tract infections cause a substantial amount of illness and death in children. Respiratory syncytial virus (RSV), influenza A and B viruses, parainfluenza viruses, human adenoviruses, rhinoviruses, coronaviruses, and the more recently identified human metapneumovirus (HMPV) all cause respiratory tract infections. However, in a substantial proportion of respiratory tract infections, no etiologic agent is detected (*1*), which suggests unknown pathogens. A previously unknown virus likely involved in children's respiratory tract infections has been recently described in Sweden (*2*) and was also identified in Australia (*3*) and Japan (*4*). This newly identified virus shares a high sequence identity and a similar genomic organization with bovine parvovirus and canine minute virus, 2 related members of the *Bocavirus* genus in the *Parvovirinae* subfamily of the *Parvoviridae* family, and it was provisionally named human bocavirus (HBoV).

To investigate epidemiologic features of this virus and further specify clinical signs associated with HBoV infections, we retrospectively tested respiratory specimens from children obtained during a 1-year period. We report the incidence and seasonal distribution of HBoV, provide a phylogenetic analysis of HBoV isolates, and describe clinical characteristics of HBoV infection.

The Study

The study sample comprised 589 children <5 years of age who were admitted to a pediatric unit of the University Hospital of Montpellier (France) for acute respiratory tract disease, from November 2003 to October 2004. They were 306 boys and 283 girls with a median age of 7 months (range 2 days–60 months). Nasopharyngeal aspirates from these children were tested for common viral respiratory pathogens as previously described (*5*). Samples were tested for respiratory viruses by direct immunofluorescence assays with monoclonal antibodies to RSV; influenza A and B viruses; parainfluenza type 1, 2, and 3 viruses; and human adenovirus. Samples were also injected into MRC5 cell monolayers for virus isolation, and they were tested for HMPV by reverse transcription PCR. Aliquots of samples and of nucleic acid extracts were stored at –80°C.

Nucleic acid extracts were tested for HBoV DNA by PCR with primers targeting the predicted *NP1* gene (*2*). A negative control was included in each PCR run. All HBoV-positive samples were quantitated by real-time PCR with a second sample aliquot. For quantitating HBoV DNA in the positive samples, the 354-bp NP1 PCR fragment was cloned into pGEM-T Easy Vector (Promega, Charbonnières, France). The obtained HBoV-NP1 plasmid was used as control in a subsequent 5′-exonuclease-based real-time PCR assay performed on a LightCycler 2.0 (Roche Diagnostics, Meylan, France) with 2 inner primers (BocaRT1, 5′-CGAAGATGAGCTCAGGGAAT-3′ and BocaRT2, 5′-GCTGATTGGGTGTTCCTGAT-3′) and a FAM/TAMRA dually labeled probe (5′-FAM-CACAGGAGCAGGAGCCGCAG TAMRA-3′). Amplification was performed on 10 μL nucleic acid extract with 0.5 μmol/L both primers and probe and 3 mmol/L $MgCl_2$ with FastStart DNA Hybridization Mix (Roche Diagnostics). For quantitation, standard curves were generated by 10-fold dilutions of the pGEM-T HBoV NP1 plasmid. Sensitivity of the PCR assay was 50 copies per reaction as determined by dilutions of the plasmid. DNA level in the HboV-positive samples was measured by using the LightCycler control DNA kit (Roche Diagnostics). Results were expressed as log HBoV DNA copies per nanogram of extracted DNA.

We identified 268 viruses in 259 (44.0%) of the 589 children. Of the 589 children, 165 (28.0%) were infected with RSV, 50 (8.5%) with HMPV, 18 (3.1%) with influenza A viruses, 18 (3.1%) with rhinoviruses, 9 (1.5%) with parainfluenza type 3 viruses, and 8 (1.3%) with human adenoviruses.

Nasopharyngeal aspirates were positive for HBoV in 26 children (4.4%). Among these HBoV-infected children, 9 (34.6%) were coinfected with another respiratory virus: 5 with RSV, 2 with HMPV, and 2 with human adenovirus. The median HBoV viral load was 2.61 log copies/ng of DNA (range 1.26–4.26 log copies/ng of DNA). The seasonal distribution of respiratory viruses is shown in Figure 1; HBoV was detected from December to June.

Seven HBoV isolates (corresponding to 1 isolate per month) were selected for sequence analysis of a 1-kb DNA fragment encompassing the *VP2* gene. Amplification and sequencing primers were as follows: BocaSEQ1 (5′-AAAATGAACTAGCAGATCTTGATG-3′), BocaSEQ4 (5′-GAACTTGTAAGCAGAAGCAAAA 3′), BocaSEQ2 (5′-GTCTGGTTTCCTTTGTATAGGAGT-

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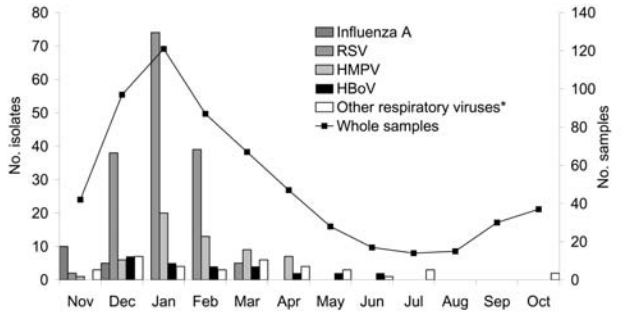

Figure 1. Seasonal distribution of infections caused by human bocavirus (HBoV), human metapneumovirus (HMPV), respiratory syncytial virus (RSV), influenza A virus, and other respiratory viruses during the 1-year study period (November 2003–October 2004). *Other viruses include parainfluenza virus type 3, human adenoviruses, and picornaviruses.

3′), and BocaSEQ3 (5′-GACCCAACTCCTATACAAAG-GAAAC-3′). These HBoV VP2 sequences were deposited in GenBank under accession numbers AM160609 to AM160615. The nucleotide sequences were aligned, and a phylogenetic tree was constructed to include the 2 previously deposited HBoV sequences ST1 and ST2 as well as the VP2 sequences of canine minute virus (NC_004442) and bovine parvovirus (NC_001540). The VP2 gene of human parvovirus B19 (NC_000883) was chosen as outgroup to root this tree (Figure 2). Our VP2 sequences shared 97.5%–100% nucleotide identity with the HBoV prototype strains ST1 and ST2, whereas amino acid identity was 98.9%–100%.

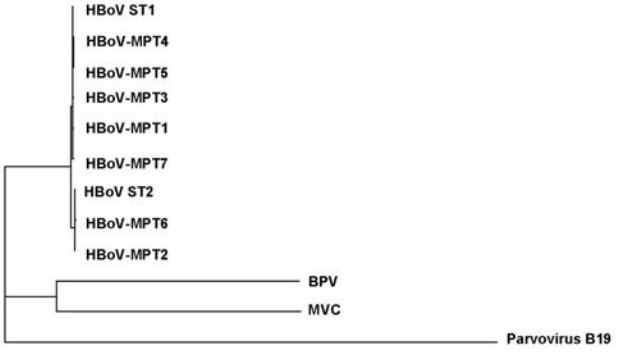

Figure 2. Phylogenetic analysis of human bocavirus (HBoV) VP2 sequences. Viruses detected in the present study are prefixed HBoV-MPT, followed by the isolate number (GenBank accession nos. AM160609 to AM160615). The nucleotide sequences were aligned with the ClustalW software. Phylogenetic trees were constructed by neighbor-joining through the Institut Pasteur website (http://www.pasteur.fr) with the DNADist and neighbor-joining software packages of the PHYLIP program. The default transition to transversion ratio of 2.0 was retained. We computed 100 bootstrap datasets with random sequence addition to generate consensus trees. BPV, bovine parvovirus; MVC, canine minute virus (minute virus of canine).

The 26 HBoV-infected children had a median age of 13 months, and the ratio of boys to girls was 1.9. The median duration of hospital stay was 4 days (range 2–39 days). Bronchiolitis was the leading diagnosis, and upper respiratory pathologic features were uncommon. The clinical signs and symptoms among children with HBoV are shown in the Table. The predominant symptoms were dyspnea, respiratory distress, and cough. Half of the children had fever (temperature >38°C). Chest radiographs were obtained for 18 HBoV-infected children, and 15 of them (83.3%) showed abnormal findings, such as hyperinflation or interstitial infiltrates. History of asthma or previous bronchiolitis episodes were reported in 6 HBoV-infected children (23.0%). Three children (11.5%) had an underlying disease (1 congenital heart disease and 2 chronic respiratory diseases), and 7 (26.9%) were born preterm (<36 weeks). Laboratory parameters such as oxygen saturation, C-reactive protein level, or leukocyte count were not relevant in this context.

Table. Characteristics of 26 children infected with HBoV*

Characteristic	Value
Demographic data	
Age (mo), median (range)	13 (4–43)
No. boys/no. girls	17/9
Virologic data	
HBoV viral load (log copies/ng DNA), median (range)	2.61 (1.26–4.26)
Viral coinfection,† no. (%)	9 (34.6)
Laboratory findings	
CRP (mg/L), median (range), n = 22	13.5 (<5–166)
Leukocytes ($\times 10^3/\mu L$), median (range), n = 24	13.2 (7.7–32.0)
SaO_2 (%), median (range), n = 17	93 (68–99)
Chest radiographic findings, no. (%), n = 18	
Hyperinflation	14 (72.2)
Infiltrate	7 (38.8)
Atelectasis	2 (11.1)
Normal	3 (16.6)
Clinical findings, no. (%)	
Temperature >38°C	13 (50.0)
Cough	13 (50.0)
Dyspnea, wheezing	14 (53.8)
Respiratory distress	14 (53.8)
Rhinorrhea, pharyngitis	8 (30.7)
Otitis	4 (15.4)
Final diagnosis, no. (%)	
Bronchiolitis	12 (46.1)
Pneumonia	3 (11.5)
Asthma	7 (26.9)
Upper respiratory tract infection	4 (15.4)

*HBoV, human bocavirus; CRP, C-reactive protein; SaO_2, arterial oxygen saturation.
†Respiratory syncytial virus (n = 5), human metapneumovirus (n = 2), and adenovirus (n = 2).

Conclusions

Our data indicate that among children <5 years of age hospitalized with a community-acquired respiratory tract infection, HBoV was detected in 4.4% and represented the third most likely etiologic agent after RSV and HMPV. This 4.4% incidence is in accordance with the values of 3.1%, 5.6%, and 5.7% reported by Allander et al. (*2*), Sloots et al. (*3*), and Ma et al. (*4*). HBoV was detected from December to June, which suggests an epidemiologic difference with respiratory viruses such as RSV, HMPV, or influenza A virus (Figure 1). The absence of HBoV circulation during summer and early fall needs further confirmation. Indeed, this finding might be in part explained by the low number of samples collected during this period.

We previously showed that the HBoV *NP1* gene displayed limited sequence variation (*6*). In the present study, we also observed only minor sequence variations in the *VP2* gene, which suggests that the diverse strains of HBoV belong to a unique lineage. This low genetic diversity of HboV was also reported by Sloots et al., who analyzed the *NS1* gene (*3*).

HBoV infections were mainly identified in children with lower respiratory tract diseases such as bronchiolitis. HBoV was also found in children with asthma exacerbation and, less frequently, in children with upper respiratory infection, which suggests that HBoV shares clinical features with respiratory viruses such as RSV or HMPV (*5*,*7*). Underlying diseases, asthma, or previous bronchiolitis episodes as well as history of prematurity were observed in 61.5% of HBoV-infected children. Even though this feature may be explained by more systematic hospitalization of these children, HBoV pathogenicity may be facilitated by predisposing conditions.

In 9 (34.6%) instances, HBoV was detected concurrently with another respiratory virus. In the original description of HBoV, 17.6% of coinfections involved human adenovirus or RSV (*2*). More recently, a high rate of coinfection (55.6%) was seen in a population of children ≤3 years of age (*3*). Considering these high rates of coinfection, the exact role played by HBoV in respiratory tract diseases needs to be more precisely defined.

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References

1. Juven T, Mertsola J, Waris M, Leimonen M, Meurman O, Roivanen M, et al. Etiology of community-acquired pneumonia in 254 hospitalized children. Pediatr Infect Dis J. 2000;19:293–8.
2. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Anderson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005;102:12891–6.
3. Sloots TP, McErlean P, Speicher DJ, Arden K, Nissen MD, Mackay IA. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol. 2005;35:99–102.
4. Ma X, Endo R, Ishiguro N, Ebihara T, Ishiko H, Ariga T, et al. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol. 2006;44:1132–4.
5. Foulongne V, Guyon G, Rodière M, Segondy M. Human metapneumovirus infection in young children hospitalized with respiratory tract disease. Pediatr Infect Dis J. 2006;25:354–9.
6. Foulongne V, Rodière M, Segondy M. Human bocavirus in children. Emerg Infect Dis. 2006;12:862–3.
7. Iwane MK, Edwards KM, Szilagyi PG, Walker FJ, Griffin MR, Weinberg GA, et al. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. Pediatrics. 2004;113:1758–64.

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Bocavirus Infection in Hospitalized Children, South Korea

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This study presents the first evidence of human bocavirus infection in South Korean children. The virus was detected in 27 (8.0%) of 336 tested specimens, including 17 (7.5%) of 225 virus-negative specimens, collected from children with acute lower respiratory tract infection.

Human respiratory syncytial virus (HRSV), human metapneumovirus (HMPV), parainfluenzavirus, coronavirus, and adenovirus are commonly detected viruses in children with acute lower respiratory tract infections (LRTI) (*1–5*). Recently, human bocavirus (HBoV) was identified as a cause of human respiratory tract infections in Sweden (*6*). Although hBoV was initially suspected to be an important human respiratory pathogen, the worldwide prevalence and clinical significance of the infection are still unclear. The purpose of this study was to investigate the prevalence of HBoV infection in children hospitalized with acute LRTI in South Korea.

The Study

A total of 336 specimens were evaluated for the presence of HBoV, including 225 virus-negative specimens (median age of patients = 14 months; range 1–69 months) and 111 virus-positive specimens (median age of patients = 15 months; range 1–83 months). The virus-positive specimens comprised 90 specimens with HRSV, 19 with HMPV, and 2 with adenovirus and were taken from hospitalized children with LRTI at Sanggyepaik Hospital from July 2004 through October 2005. LRTI included the diagnoses of bronchiolitis, bronchitis, pneumonia, and laryngotracheobronchitis. The 336 nasopharyngeal samples were consecutively collected at admission, after informed consent was obtained from the children's parents. Common respiratory viruses (HRSV, adenovirus, influenza A and B, parainfluenza) were detected by direct fluorescent-antibody assay kit (Dako Imagen, Cambridgeshire, UK), after admission. The remaining samples were stored at –70°C for further studies. Viral RNA was extracted from each sample by a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), and reverse transcription of 0.5 μg of each RNA sample was performed. Reverse transcription–PCR was performed to detect HMPV by using F-gene primers (*7*) and human coronavirus NL-63, by using a 1-a and a 1-b primer, as previously described (*8*).

DNA was extracted from the nasopharyngeal aspirates with a QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Two PCR assays were performed for each sample, 1 for the NP1 gene and the other for the NS1 gene, using different primer sets;188F (5′-GACCTCTG-TAAGTACTATTAC-3′) and 542R (5′-CTCTGT-GTTGACTGAATACA G-3′) for the NP1 gene; and HBoV01.2 (5′-TATGGCCAAGGCAATCGTCCAAG-3′) and HBoV02.2 (5′-GCCGCGTGAACATGAGAAACA-GA-3′) for the NS1 gene, as previously described (*6*,*9*). Each cycle comprised predenaturation at 95°C for 3 min and 35 amplification cycles (denaturation at 95°C for 1 min, primer annealing [at 54°C for NP1 gene and 56°C NS1 gene] for 1 min, and extension at 72°C for 1 min). The amplified DNA fragments for the NP1 gene and NS1 gene were 354 bp and 291 bp, respectively. To validate the amplification process and exclude carryover contamination, positive and negative controls were run for each PCR, and positive samples were verified against an independent RNA extraction.

Amplicon was purified by using QIAquick (Qiagen GmbH) and sequenced in both directions with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing products were resolved with an ABI 3730 XL autoanalyzer (Applied Biosystems). Nucleotides sequences were aligned with BioEdit v7.0 and presented in a topology tree, prepared in MEGA 3.1 (*10*).

Conclusions

This study presents the first evidence of HBoV infection in Korean children, which suggests that HBoV can infect humans worldwide (*1*,*2*). In this study, HBoV was detected in 27 (8.0%) of 336 tested specimens and in 17 (7.5%) of 225 virus-negative specimens collected from children with acute LRTI. A total of 15 (55.6%) of 27 HBoV-positive specimens were obtained from <1-year-old children, 8 (29.6%) of 27 1- to 2-year-old children, and 4 (14.8%) of 27 3- to 5-year-old children. Other viruses were also detected in 10 (37%) of 27 HBoV-positive specimens; 5 specimens also contained HRSV, 4 had HMPV, and 1 had adenovirus. Although HBoV was detected mostly in the winter in previous studies (*6*,*9*), we detected it throughout the study period.

The medical records of 17 patients with samples positive for only HBoV were retrospectively reviewed. The patients ranged in age from 1 to 37 months of age, and the

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male-to-female ratio was 2.8:1.The clinical manifestations of HBoV-positive patients were fever (76.4%), cough (76.4%), rhinorrhea (23.5%), gastrointestinal symptoms (11.7%), and rashes (5.8%). Direct sequencing of PCR products of the NS1 gene and NP1 genes showed that most strains had the same sequences (Figures 1 and 2). The prevalence of HBoV in this study was higher than that in previous studies (3.14%–5.7%). This variation may be due to differences in the characteristics of the study populations and collection time of respiratory specimens (*6*,*9*,*11*). In our study, the positive rates of HBoV in the nasopharyngeal aspirates of patients with acute LRTI were relatively high. However, the clinical role of HBoV is still unclear because of a high frequency of co-infection (37%), a finding similar to those of previous studies (17.6%–55.6%) (*6*,*9*). In a future study, additional microbial testing for rhinovirus and coronaviruses, which are known to cause LRTIs, is needed to evaluate the precise rate of detection of ≥1 virus (*12*,*13*).

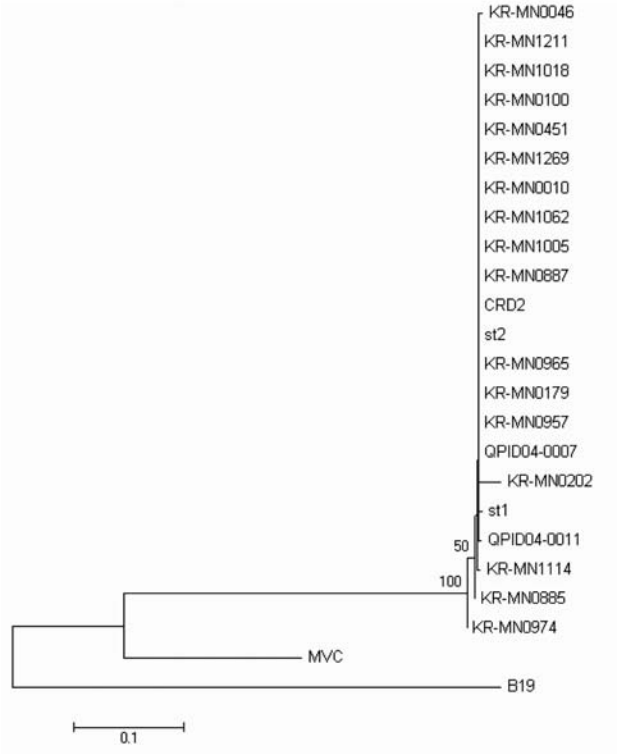

Figure 1. Phylogenetic analysis of Korea (KR-MN), Sweden (st), USA (CRD2), and Queensland (Q) NS1 gene sequences from human bocavirus strains presented on a topology tree prepared in MEGA3.1. Nucleotide alignment of a 245-bp portion of the NS1 gene was prepared by using BioEdit v7.0. The nucleotide distance matrix was generated with Kimura 2-parameter estimation. Nodal confidence values indicate the results of bootstrap resampling (n = 1,000). GenBank accession no. B19 (human erythrovirus B19, DQ408301); MVC (canine minute virus, NC_004442); st1 (HBoV strain st1, DQ000495); st2 (HBoV strain st2, DQ000496); QPID04-0007 (DQ200648); QPID04-0011 (DQ206702); CRD2 (DQ340570).

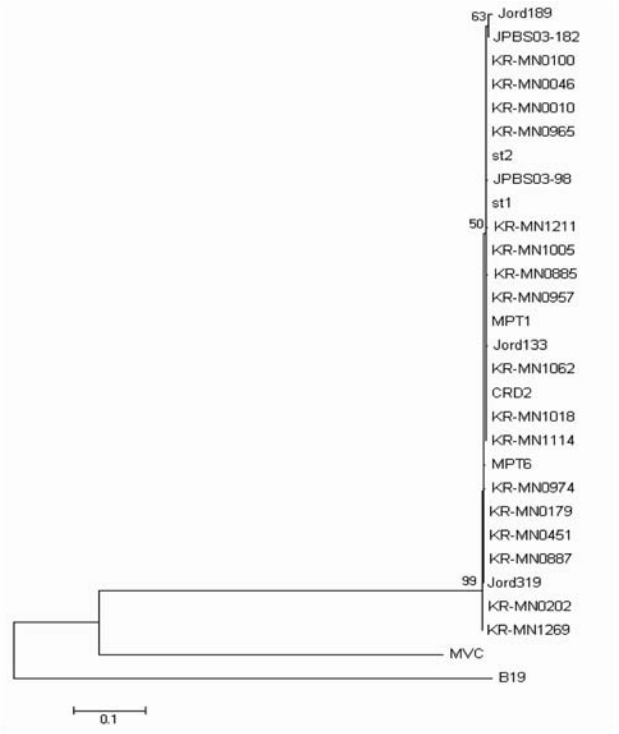

Figure 2. Phylogenetic analysis of Korean (KR-MN), Swedish (st), Jordanian (Jord), French (MP), USA (CRD2), and Japanese (JPBS) NP1 gene sequences from human bocavirus strains presented on a topology tree prepared in MEGA3.1. Nucleotide alignment of a 310-bp portion of the NP1 gene was prepared by using BioEdit v7.0. The nucleotide distance matrix was generated with Kimura 2-parameter estimation. Nodal confidence values indicate the results of bootstrap resampling (n = 1,000). GenBank accession nos. B19 (human erythrovirus B19, DQ408301); MVC (canine minute virus, NC_004442); st1 (HBoV strain st1, DQ000495); st2 (HBoV strain st2, DQ000496); Jord37 (AB243566); Jord133 (AB243567); Jord189 (AB243568); Jord319 (AB243570); JPBS03-98 (DQ296618); JPBS03-182 (DQ296620); JPBS05-52 (DQ296635); MPT1 (AM109958); MPT6 (AM109964); CRD2 (DQ340570).

In conclusion, we confirmed HBoV infection in hospitalized children with acute LRTI in South Korea. Further prospective population-based studies are needed to confirm the role of HBoV in LRTI in children.

Dr Chung is a researcher at the Sanggyepaik Hospital, Inje University College of Medicine, South Korea. His research interests include viruses that cause respiratory tract infections in children and the molecular epidemiology of HMPV and HCoV NL-63.

References

1. Selwyn BJ. The epidemiology of acute respiratory tract infection in young children; comparison of findings from several developing countries. Rev Infect Dis. 1990;12:S870–88.

2. Hall CB. Respiratory syncytial virus and parainfluenza virus. N Engl J Med. 2001;344:1917–28.
3. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med. 2001;7:719–24.
4. Boivin G, Abed Y, Pelletier G, Ruel L, Moisan D, Cote S, et al. Virological features and clinical manifestations associated with the human metapneumovirus, a new paramyxovirus responsible for acute respiratory tract infections in all age groups. J Infect Dis. 2002;186:1330–4.
5. van der Hoek L, Sure K, Ihorst G, Stang A, Pyrc K, Jebbink MF, et al. Croup is associated with the novel coronavirus NL63. PLoS Med. 2005;2:e240.
6. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005;102:12891–6.
7. van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, de Swart RL, et al. Antigenic and genetic variability of human metapneumoviruses. Emerg Infect Dis. 2004;10:658–69.
8. Arden KE, Nissen MD, Sloots TP, Mackay IM. New human coronavirus, HCoV-NL63, associated with severe lower respiratory tract disease in Australia. J Med Virol. 2005;75:455–62.
9. Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, MacKay IM. Evidence of human coronavirus HKU-1 and human bocavirus in Australian children. J Clin Virol. 2006;35:99–102.
10. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2; molecular evolutionary genetics analysis software. Bioinformatics. 2001;17:1244–5.
11. Ma X, Endo R, Ishiguro N, Ebihara T, Ishiko H, Ariga T, et al. Detection of human bocavirus in Japanese children with lower respiratory tract infections. J Clin Microbiol. 2006;44:1132–4.
12. Hayden FG. Rhinovirus and the lower respiratory tract. Rev Med Virol. 2004;14:17–31.
13. van Elden LJ, van Loon AM, van Alphen F, Hendriksen KA, Hoepelman AI, van Kraaij MG, et al. Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcriptase polymerase chain reaction. J Infect Dis. 2004;189:652–7.

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Changing Pattern of Visceral Leishmaniasis, United Kingdom, 1985–2004

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A 20-year (1985–2004) retrospective review of 39 patients with imported visceral leishmaniasis found that tourism to Mediterranean countries and HIV infection were associated with visceral leishmaniasis. Diagnosis was often delayed. Treatment with liposomal amphotericin B has improved prognosis. Visceral leishmaniasis should be made a reportable disease.

Each year, 500,000 new cases of visceral leishmaniasis (VL) are reported worldwide. The number of cases and endemic foci for VL have increased during the past 2 decades (*1–4*). These increases may be the result of improved detection and surveillance methods, or they may be actual increases in numbers, possibly driven by increasing rates of HIV infection (*5*,*6*). VL is a well-recognized but uncommon imported disease in the United Kingdom, but it is not reportable, so information on its importation is incomplete. Data for this report (e.g., date of confirmation of infection, country where acquired, HIV status) were collated and supplied by the Travel Health Surveillance Section of the Health Protection Agency, Communicable Disease and Surveillance Centre, United Kingdom.

The Study

UK patients with VL are often referred to the Hospital for Tropical Diseases, London, for confirmation of diagnosis or treatment. We reviewed all cases of VL seen at this hospital from 1985 through 2004. Thirty-nine patients were identified from our hospital database and laboratory records, representing 83% of cases reported in the United Kingdom (Figure 1).

The mean age of these patients was 36 years (range 2–66 years); 4 patients were <15 years of age. The male:female ratio was 2:1. Patients acquired visceral leishmaniasis while in the following areas: 30 (76.9%) in Mediterranean countries (13 in Spain, 9 in Italy, 4 in Greece, 3 in Malta, 1 in Cyprus), 5 (12.8%) from Africa, 3 (7.7%) from Asia, and 1 (2.6%) from South America. During the entire 20-year period, 55.5% of patients had been tourists to these VL-endemic regions, and 44.5% were immigrants or refugees, but after 2000, all patients were tourists. One third of the patients were HIV-antibody positive. Two patients had significant immunosuppression from other causes: 1 from chronic lymphatic leukemia and 1 from immunosuppressive drugs received after kidney transplantation.

Time from onset of symptoms to diagnosis was 1–11 months (mean 3 months). Diagnoses of VL were confirmed by ≥1 method: microscopic identification of amastigotes in tissue aspirates; histologic examination of biopsy material from bone marrow, liver, or spleen; serologic analysis; and PCR for leishmania DNA (*7*). Leishmania amastigotes were found in 32 bone marrow aspirates, 3 liver biopsy specimens, 2 splenic aspirates, and 1 skin biopsy specimen. PCR to detect leishmania DNA has been performed in this study since 1995 and was positive in 7 of 11 cases. It was the method of confirmatory diagnosis for 1 patient (performed on a bone marrow aspirate that had no visible amastigotes). Serum analysis for leishmania antibodies was performed on samples from 33 patients; results were positive for all HIV-negative patients and for 2 of 13 HIV-positive patients (Table 1).

Before 1995, treatment of leishmaniasis involved several drugs, including sodium stibogluconate, paromomycin, meglumine antimoniate, and pentamidine. The latter 2 were used for patients who received initial treatment outside the United Kingdom; within the United Kingdom, most (59%) patients were treated with sodium stibogluconate. After 1995, liposomal amphotericin became the drug of choice and was used in 14 (83%) of 17 patients. HIV status did not affect drug choice.

Figure 2 shows the frequency of relapses with each drug treatment in our cohort. Of the 13 patients who had a relapse, 8 (53%) had HIV coinfection. Of the 13 who had

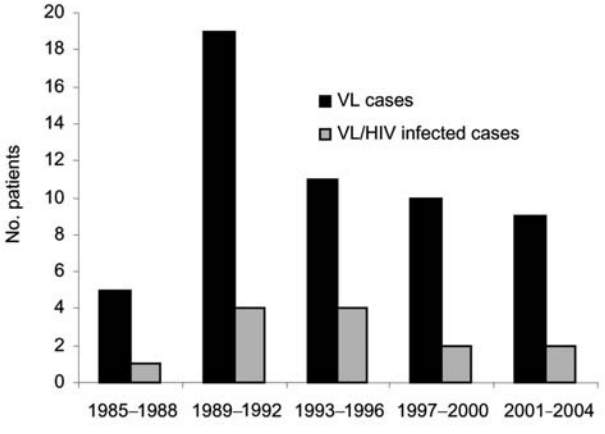

Figure 1. Number of visceral leishmaniasis (VL) cases, United Kingdom, 1985–2004 (data from Health Protection Agency).

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Table 1. Serologic testing in patients with and without HIV infection*

	IFAT		DAT		LATEX		k39	
VL cases (no. patients)	+	–	+	–	+	–	+	–
HIV negative (20)	18	0	20	0	5	0	4	0
HIV infected (13)	1	8	0	9	0	1	1	0

*VL, visceral leishmaniasis; IFAT, indirect immunofluorescent antibody test, 1985–1994; DAT, direct antibody agglutination test, beginning in 1989; LATEX, latex agglutination test, 1996–2003; k39, recombinant k39 antigen detection test, beginning in 2002; +, positive result; –, negative result.

HIV coinfection, 8 (62%) had a relapse, compared with 7 (27%) of the 26 HIV-negative patients who had a relapse. Two patients had other risk factors for relapse: 1 had had a kidney transplant, and the other had chronic active hepatitis B. Three patients who had relapses had no apparent risk factors. Relapse occurred in 5 (33%) of 15 patients who received sodium stibogluconate, compared with 1 (7%) of 14 who received liposomal amphotericin as their initial drug. At first, relapsed patients were treated with a combination of sodium stibogluconate and allopurinol, which was unsuccessful in contrast with its reported success in Kenya, or with amphotericin deoxycholate (*8*). HIV-infected patients who had relapses received further treatment with liposomal amphotericin, usually successful, but 2 became unresponsive to treatment. Miltefosine was used in 2 patients with HIV coinfection who had relapses, but relapses recurred, 1 while the patient was still receiving the drug (*9*). Since 2000, pentamidine prophylaxis has been used in 3 immunocompromised patients.

Table 2 summarizes outcomes. The number of patients in the final cure category excludes patients who had had a relapse. The 2 patients who refused treatment had HIV coinfection; 1 died. After 1995, the final cure rate improved and fewer patients required retreatment. The 3 patients who died had advanced HIV disease; 1 had an additional complication of liver failure secondary to hepatitis C cirrhosis.

Conclusions

The data from this cohort show that VL is imported into the United Kingdom at a constant rate, particularly in adult male tourists to the Mediterranean, and that during the past 20 years, HIV and VL had interacted strikingly. The Mediterranean VL-endemic zone accounts for only a small proportion of VL cases globally, but it is a popular holiday destination for the British, and tourism is driving the epidemiology of the imported infection. In the past 5 years, we have seen no immigrants or refugees with VL. The data show that high rates of tourism to an area of low endemicity for a parasitic disease can result in a substantial number of imported cases.

One third of our patients were immunocompromised. The highest rates of reported HIV coinfection worldwide are from Europe, where 85% of the cases are from the southwest; 71% of these are in intravenous drug users (*2*). HIV/VL coinfection is a serious disease that has high death rates, reflected by the 3 deaths in our cohort. These patients had late-stage HIV disease and additional coexisting conditions. HIV/VL-coinfected patients have higher relapse rates and decreased life expectancy. Highly active antiretroviral therapy for HIV is decreasing the numbers of these coinfections and improving survival rates (*10*,*11*).

The mean time from symptom onset to diagnosis was 3 months. This delay resulted mainly from physicians' failure to consider the diagnosis, although a few cases were difficult to diagnose. For 2 patients, initial microscopic examination of bone marrow biopsy results did not show amastigotes; reinspection showed scarce amastigotes. For another patient, serologic results by direct agglutination were negative when performed early in the course of disease but positive when repeated. The low sensitivity of serologic tests for HIV-infected patients in this cohort confirms that serologic tests cannot be relied on as a means of excluding VL in HIV-infected patients.

Our cohort shows a rise and fall in the proportion of cases of HIV/VL coinfection, starting in the early 1990s, rising to a peak of 50% during 1993–1996, and then decreasing, possibly because of greater use of highly active antiretroviral therapy. Official UK data on VL are incomplete and do not accurately reflect trends. VL remains an imported disease in the United Kingdom and is often associated with HIV infection. We suggest that a for-

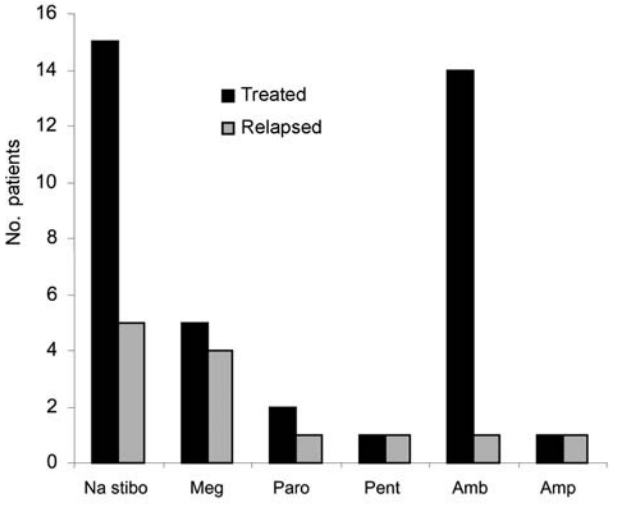

Figure 2. Initial drug treatment for patients with visceral leishmaniasis and number of relapses. Na stibo, stibogluconate; Meg, meglumine antimoniate; Paro, paromomycin; Pent, pentamidine; Amb, ambisome; Amp, amphotericin.

Table 2. Patient response to treatment and outcome

Years	No. patients	Initial cure or response to treatment (%)	Refused treatment (%)	Final cure (%)	Died while receiving treatment (%)
1985–1994	22	7 (35)	2 (9)	18 (90)	2 (9)
1995–2004	17	13 (76)	0	16 (94)	1 (6)

mal notification system for visceral leishmaniasis in the United Kingdom, as elsewhere in Europe, would be beneficial for monitoring trends and health planning (*12*). Our data also show the importance of looking at rare imported diseases over a long period so that emerging risk factors can be identified. These data also highlight the usefulness of having 1 center that deals with unusual infections, where expertise in diagnosis and management can be built up and maintained.

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Dr Malik was senior house officer at the Hospital for Tropical Diseases when she became interested in VL. She is now pursuing a career in ophthalmology.

References

1. Desjeux P. The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg. 2001;95:239–43.
2. Gabutti G, Balestra G, Fkego G, Crovari P. Visceral leishmaniasis in Liguria, Italy. Lancet. 1998;351:1136.
3. Punda-Polic V, Sardelic S, Bradaric N. Visceral leishmaniasis in southern Croatia. Lancet. 1998;351:188.
4. World Health Organization. Leishmaniasis and leishmania/HIV coinfection. WHO report on global surveillance of epidemic-prone infectious diseases 2000. Report no. WHO/CDS/CSR/ISR/2000.1. Geneva: The Organization; 2000.
5. Guerin PJ, Olliaro P, Sundar S, Boelaert M, Croft SL, Desjeux P, et al. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. Lancet Infect Dis. 2002;2:494–501.
6. Alvar J, Canavate C, Guiterrez-Solar B, Jimenez M, Laguna F, Lopez-Velez R, et al. *Leishmania* and human immunodeficiency virus co-infection: the first 10 years. Clin Microbiol Rev. 1997;10:298–319.
7. Noyes HA, Reyburn H, Bailey W, Smith D. A nested PCR based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. J Clin Microbiol. 1998;36:2877–81.
8. Chunge CN, Gachini G, Muigai R, Wasunna K, Rashid JR, Chulay JD, et al. Visceral leishmaniasis in response to antimonial drugs. III. Successful treatment using a combination of sodium stibogluconate plus allopurinol. Trans R Soc Trop Med Hyg. 1985;79:715–8.
9. Sindermann H, Engel KR, Fischer C, Bommer W. Oral miltefosine for leishmaniasis in immunocompromised patients: compassionate use in 39 patients with HIV co-infection. Clin Infect Dis. 2004;39:1520–3.
10. Pagliano P, Rossi M, Rescigno C, Altieri S, Coppola MG, Gramiccia M, et al. Mediterranean visceral leishmaniasis in HIV negative adults: a retrospective analysis of 64 consecutive cases (1995–2001). J Antimicrob Chemother. 2003;52:264–8.
11. Russo R, Laguna F, Lopez-Velez R, Medrano FJ, Rosenthal E, Cacopardo B, et al. Visceral leishmaniasis in those infected with HIV: clinical aspects and other opportunistic infections. Ann Trop Med Parasitol. 2003;97:99–105.
12. Harms G, Schonian G, Feldmeier H. Leishmaniasis in Germany. Emerg Infect Dis. 2003;9:872–5.

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Mental Status after West Nile Virus Infection

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Mental status after acute West Nile virus infection has not been examined objectively. We compared Telephone Interview for Cognitive Status scores of 116 patients with West Nile fever or West Nile neuroinvasive disease. Mental status was poorer and cognitive complaints more frequent with West Nile neuroinvasive disease (p = 0.005).

West Nile virus (WNV) outbreaks have been studied in Africa since 1937 and in the United States since the initial New York City outbreak in 1999 (*1*). Studies of these outbreaks typically include only hospitalized patients, use retrospective medical chart reviews, and do not include follow-up after discharge (*1*–*5*). Therefore, the long-term sequelae of WNV are largely unknown.

Mental status after West Nile virus infection is an important public health issue because many studies of hospitalized patients have reported continued complaints from the time of discharge (*2*,*4*,*5*) through 18 months later (*6*). A limitation of these studies is their reliance on self-report of cognitive deficits rather than objective examination. No study of WNV patients has used objective assessment of mental status to determine the severity of cognitive deficits after acute WNV infection in a large sample of WNV patients, despite the fact that altered mental status is reported in 46% (*1*,*4*) to 74% (*3*) of WNV patients at the time of discharge from the hospital. In addition, no studies have determined whether mental status changes are more prevalent in patients who had West Nile neuroinvasive disease (WNND) than in patients who had West Nile fever (WNF), which would be expected, given the diagnostic criteria and the reports of less frequent and less severe cognitive deficits from WNF patients (*7*).

The purpose of our study was to objectively compare mental status of patients with a diagnosis of WNND or WNF, 9 months after symptom onset. We used the Telephone Interview for Cognitive Status (TICS) and subjective cognitive complaints noted during interview. Of the 190 eligible patients, all were seropositive for WNV and all had been reported to the New Mexico State Department of Health in 2003 (*8*). We successfully contacted 129 (68%) of these patients by telephone and excluded 13 who had received a diagnoses of a neurologic condition before the diagnosis of WNV infection or who did not speak English well. For the remaining 116 patients, diagnosis of WNND or WNF was made without knowledge of TICS score and was based on the reporting physician's diagnosis or medical record review for patients who were hospitalized for WNV infection or who had continuing neurologic or cognitive symptoms at the time of interview. Patients were evaluated with the TICS, which is highly correlated with the Mini Mental Status Examination (*9*), is sensitive to mental status deficits in the elderly (*10*,*11*), and is standardized for administration by telephone (*12*).

Table 1 shows that the WNF and WNND groups were comparable in age, sex, and ethnicity (p>0.05). However, because of a trend for lower education in the WNND group (p = 0.05), education was a covariate in all analyses. Analysis of covariance (ANCOVA) showed TICS total score to be poorer for the WNND than the WNF group (p = 0.005). Thus, a small, but consistent, effect suggests that WNV infection severity affects mental status.

Participants were also asked questions about current cognitive functioning (Table 2). Frequency of self-report of cognitive problems varied from 6% to 42% across both groups. Logistic regression, when controlled for education, showed reports of concentration difficulty (p = 0.05) and confusion (p = 0.02) to be significantly higher in the WNND group. Overall, the WNND group reported more cognitive problems than the WNF group (1.6 vs. 0.8, respectively, p = 0.009), and the number of cognitive problems was correlated with the TICS total score (r = –0.21,

Table 1. Demographic data from Telephone Interview for Cognitive Status*†

Characteristic	West Nile fever, n = 64	West Nile neuroinvasive disease, n = 52	p value
Age, y	50.0 (12.8)	53.6 (19.1)	0.26
Education, y	14.6 (2.6)	13.6 (3.0)	0.05
Sex, % male	44	58	0.14
Ethnicity			
% white	62	64	0.90
% Hispanic	33	31	0.90
Hospitalized, n (%)	10 (16)	40 (78)	<0.001
TICS total (range 0–41)	33.6 (3.3)	31.1 (3.8)	0.005‡

*TICS, Telephone Interview for Cognitive Status.
†Means with standard deviations in parentheses, except where otherwise indicated.
‡p value, after analysis of covariance, controlling for marginal group difference in education.

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Table 2. Percentage of patients reporting current cognitive problems

Mental deficit	West Nile fever	West Nile neuroinvasive disease	Overall
Concentration	22	42*	31
Memory	28	42	35
Understanding	11	27	18
Decision making	16	25	20
Confusion	6	25*	15
Mean rate of complaints	17	32	24

*$p \leq 0.05$, after logistic regression, controlling for marginal group difference in education.

p = 0.02). These findings indicate that self-reported cognitive problems increased with severity of WNV infection.

To determine the better predictor of WNV diagnostic category—TICS total score, rate of cognitive problems, or a combination—we performed a logistic regression predicting WNV diagnostic category from TICS total and rates of self-report of cognitive problems. Only the TICS total score significantly predicted WNND group membership (p = 0.01), but rate of report of cognitive problems was a marginal predictor (p = 0.07).

This is the first study to objectively measure mental status after WNV infection except for 1 review paper that mentioned a study that performed neuropsychological evaluation of WNV patients while they were hospitalized with acute infection (*13*). We show that 9 months after infection, WNND produces subtle but consistently greater mental status deficits than WNF. These findings are consistent with those of studies that identified a high incidence of cognitive problems from WNV patients and lesser complaints from WNF patients (*7*) from time of hospital discharge through 18 months later (*2*,*4*,*6*,*14*). We found subtle cognitive deficits in the WNND group that could not be explained by demographic variables. Although these cognitive differences are subtle, they suggest that WNND produces cognitive deficits after the acute symptoms have largely dissipated. Our data may underestimate the incidence of cognitive changes associated with WNND because more sensitive comprehensive neuropsychologic evaluations were not done.

Similar to previous studies (*5*,*6*,*14*) of chronic cognitive complaints after WNV infection, our study showed a high incidence of cognitive complaints, although subjective self-reports can be unreliable. Our data show that although ≈24% of the WNV patients complained of cognitive problems, complaints were somewhat greater for patients in the WNND group than in the WNF group. In addition, subjective reports of cognitive problems are only marginally associated with poorer mental status. This finding further supports the need to perform objective mental status examinations, especially because normal variation in cognitive performance can be misattributed to a medical diagnosis (*15*).

We were not able to determine whether the WNF group demonstrated cognitive deficits because we did not include a healthy control group and because TICS does not have normative data for respondents <60 years of age. However, the published norms for TICS recommend a cutoff score of ≥33 for classification as "normal" and ≤25 as clearly "impaired"; only 53% of our total sample fell into the normal range, despite being younger than the age for which norms are published (*9*). Furthermore, 33% of the WNF group scored in the abnormal range, suggesting that WNF may produce cognitive deficits relative to published norms. Although the influence of demographic differences (e.g., education) between the WNF and the normative group cannot be ruled out, the high incidence of abnormal scores in the WNF group may also reflect undiagnosed neuroinvasion of WNV. However, without a demographically matched control group, this question cannot be addressed definitively. In addition, 65% of the WNND group scored in the abnormal range, consistent with our other findings that WNND is associated with chronic mental status changes.

Our study has several advantages, including objective assessment of mental status, sampling from the entire state's reported cases of WNV infection in 1 year, direct comparison between WNF and WNND groups, and inclusion of patients of minority race and ethnicity. One potential limitation is the use of the reporting physician's diagnosis, but medical records were obtained for 78% of those at greatest risk for WNND. In all instances in which diagnosis was changed on the basis of medical records, WNF diagnosis was changed to WNND. Therefore, if we misclassified patients, we are more likely to have included in the WNF group patients who should have been in the WNND group; this potential bias would have decreased group differences by lowering the WNF mental status score.

These results emphasize that objective mental status assessment is more sensitive than subjective report and suggest that future studies should assess potential mental status deficits to clarify the long-term public health consequences of WNV.

Dr Haaland is a VA research career scientist at the New Mexico VA Healthcare System and professor of psychiatry and neurology at the University of New Mexico School of Medicine.

References

1. Nash D, Mostashari F, Fine A, Miller J, O'Leary D, Murray K, et al. The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med. 2001;344:1807–14.

2. Pepperell C, Rau N, Krajden S, Kern R, Humar A, Mederski B, et al. West Nile virus infection in 2002: morbidity and mortality among patients admitted to hospital in southcentral Ontario. CMAJ. 2003;168:1399–405.
3. Jeha LE, Sila CA, Lederman RJ, Prayson RA, Isada CM, Gordon SM. West Nile virus infection: a new acute paralytic illness. Neurology. 2003;61:55–9.
4. Brilla R, Block M, Geremia G, Wichter M. Clinical and neuroradiologic features of 39 consecutive cases of West Nile virus meningoencephalitis. J Neurol Sci. 2004;220:37–40.
5. Sejvar JJ, Haddad MB, Tierney BC, Campbell GL, Marfin AA, Van Gerpen JA, et al. Neurologic manifestations and outcome of West Nile virus infection. JAMA. 2003;290:511–5.
6. Klee AL, Maidin B, Edwin B, Poshni I, Mostashari F, Fine A, et al. Long-term prognosis for clinical West Nile virus infection. Emerg Infect Dis. 2004;10:1405–11.
7. Watson JT, Pertel PE, Jones RC, Siston AM, Paul WS, Austin CC, et al. Clinical characteristics and functional outcomes of West Nile fever. Ann Intern Med. 2004;141:360–5.
8. Echevarria L, Pergam S, Goade D, Davis L, Ettestad P, Sewell M, et al. Persistence of symptoms in West Nile virus from 2003 New Mexico outbreak. Presented at the Western Regional Meeting of the American Federation for Medical Research, February 3, 2005, Carmel (CA).
9. Brandt J, Folstein MF. Telephone Interview for Cognitive Status. Lutz (FL): PAR - Psychological Assessment Resources, Inc.; 2003.
10. Lines CR, McCarroll KA, Lipton RB, Block GA. Telephone screening for amnestic mild cognitive impairment. Neurology. 2003;60:261–6.
11. Plassman BL, Newman TT, Welsh KA, Helms M, Breitner JCS. Properties of the Telephone Interview for Cognitive Status: application in epidemiological and longitudinal studies. Neuropsychiatry Neuropsychol Behav Neurol. 1994;7:235–41.
12. Colditz GA, Manson JE, Hankinson SE. The Nurses' Health Study: 20-year contribution to the understanding of health among women. J Womens Health. 1997;6:49–62.
13. Arciniegas DB, Anderson CA. Viral encephalitis: neuropsychiatric and neurobehavioral aspects. Curr Psychiatry Rep. 2004;6:372–9.
14. Burton JM, Kern RZ, Halliday W, Mikulis D, Brunton J, Fearon M, et al. Neurological manifestations of West Nile virus infection. Can J Neurol Sci. 2004;31:185–93.
15. Putnam SH, Millis SR. Psychosocial factors in the development and maintenance of chronic somatic and functional symptoms following mild traumatic brain injury. Advances in Medical Psychotherapy. 1994;7:1–22.

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Human Metapneumovirus, Australia, 2001–2004

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We examined 10,025 respiratory samples collected for 4 years (2001–2004) and found a 7.1% average annual incidence of human metapneumovirus. The epidemic peak of infection was late winter to spring, and genotyping showed a change in predominant viral genotype in 3 of the 4 years.

Human metapneumovirus (HMPV) is now recognized as a substantial cause of acute respiratory infection, particularly in children; it has phenotypic and clinical characteristics that are similar to those of respiratory syncytial virus (RSV) (*1*,*2*). HMPV is ubiquitous; it infects most children at an early age, and distinct epidemic peaks are reported in the winter months (*1*,*3*–*6*). However, many of theses studies were conducted in the Northern Hemisphere and involved samples collected during a relatively short period; few data exist from extended studies over several years that involve populations in other parts of the world.

Although 2 main HMPV types are recognized (A and B) (*1*,*3*,*7*), each with 2 subtypes (A1, A2; B1, B2) (*8*), the extent of genetic variation of circulating HMPV subtypes over time has not been extensively examined. We sought to determine the incidence and seasonal distribution of HMPV in an Australian population from 2001 to 2004 and to establish the pattern of genotype distribution during those 4 years by examining the genetic variability of the P gene in 640 of 707 HMPV-positive samples.

The Study

The necessary ethics approval for this study was obtained from the Royal Children's Hospital ethics committee. We collected nasopharyngeal aspirate (NPA) specimens from January 2001 through December 2004 from patients with acute lower respiratory tract infection in Queensland, Australia. Patients were from 3 months to 93 years of age (mean 8.2 years, median 1.37 years), and 78.1% of specimens were from children <5 years of age. Nucleic acids were extracted from 0.2 mL of each NPA specimen by using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Extracts were analyzed for HMPV sequences by reverse transcriptase PCR (*9*). For samples collected during 2001 and 2002, other viral respiratory pathogens were detected by using a direct fluorescent antibody assay (DFA) in combination with a culture-augmented DFA method (*10*). For samples collected in 2003 and 2004, these pathogens were detected by multiplex PCR (*10*).

Of 10,025 NPA specimens tested, 707 were positive for HMPV, for an overall incidence of 7.1% during the 4 years. The youngest HMPV-positive patient was 4 months old, and the oldest was 79 years. In children (<18 years of age) the incidence of virus was 7.4%, and 91.9% of HMPV-positive children were <5 years of age. The seasonal distribution of HMPV infection showed a distinctive pattern for each of the 4 years studied (Figure 1). In 2001, HMPV showed broad seasonal activity; incidence was >5% in 3 consecutive seasons (autumn, winter, and spring) and peaked at 10.6% in the spring (September–November). In 2002 and 2004, most HMPV activity was in spring, (incidence of 13.6% and 15.4%, respectively), with little evidence during autumn (March–May). In 2003, the peak incidence of 9.0% occurred in winter (June–August) and persisted into spring (5.4%). In all years, virus was present well into summer (December–February), with an incidence ranging from 2.5% to 5.2%. On examination of

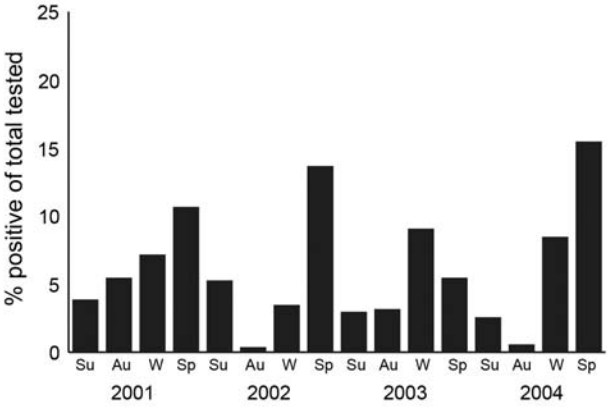

Figure 1. Seasonal incidence of human metapneumovirus, Queensland, Australia, 2001–2004. Su, summer (December–February); Au, autumn (March–May); W, winter (June–August); Sp, spring (September–November).

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those samples collected in 2003 and 2004, which were all previously analyzed for common respiratory viruses by PCR, HMPV was the most frequently detected respiratory virus in children during the spring of each year. Expressed as an annual average over the 4 years studied, the predominant viral pathogen was RSV (9.2%), followed by HMPV (7.1%), influenza A (3.5%), parainfluenza virus 3 (2.3%), and adenovirus (1.3%). In 6.8% of HMPV-positive cases, evidence of co-infection with another respiratory virus was seen; 20 patients were concurrently infected with an adenovirus, 10 with influenza A virus, 8 with RSV, 9 with parainfluenza virus 3, and 1 with parainfluenza virus 2.

Amplification products generated directly from 640 HMPV-positive NPA specimens were genotyped as previously described (*11*) (GenBank accession nos. DQ112292–DQ112320 and DQ121378–DQ121384). Data showed that all 4 viral subtypes cocirculated during each of the 4 years studied (Table 1, Figure 2). However, a different subtype predominated during 3 of the 4 years: HMPV subtype A1 was dominant in 2001, subtype A2 in 2002 and 2003, and subtype B1 in 2004 (Table 1).

Clinical records from 273 patients who were positive for HMPV were scrutinized, and data describing clinical features and length of hospital stay were recorded. Of these patients, 203 (74.4%) were admitted to hospital with a median length of admission of 3 days and a mean of 6.5 days. The predominant clinical features were cough (63%), rhinorrhea (61%), respiratory crackles/crepitations (60%), and fever (57%) (Table 2). Ninety (33.9%) of the 273 patients had a chest radiograph, and 77 (85.6%) patients showed bilateral parahilar peribronchial infiltrates consistent with a lower respiratory tract infection. Disease severity of the 273 HMPV-positive patients was classified as mild (46.8%), moderate (42.5%), and severe (10.7%), based on the use of supplemental oxygen and fluids and length of hospital stay.

Conclusions

This study of HMPV infection is the largest so far reported. The results of recent, similar studies suggested that peak periods of infection with HMPV predominate during winter in the Northern Hemisphere. However, this finding has not been extensively examined over an extended period with a large, continuous sample. Our study found that the peak period of HMPV infection in Queensland, Australia, occurs predominantly in spring (August–October) but that HMPV can be detected in every month. This finding suggests that HMPV activity, like RSV activity, occurs in the community throughout the year, and peaks of infection are a result of seasonal environmental factors.

Although RSV predominated in all years, HMPV was the second most frequently detected virus in each year studied. The low rate of co-infection of HMPV with other respiratory viruses (including RSV) suggests that co-infection may not be common in our community. When analyzing disease severity in this sample with a 2-sided test of

Table 1. Distribution of human metapneumovirus (HMPV) subtypes in Queensland, Australia, 2001–2004

Year	Total samples tested	HMPV subtype			
		A1	A2	B1	B2
2001	59	58	24	8	10
2002	122	20	51	12	17
2003	189	10	36	30	24
2004	270	1	23	59	17

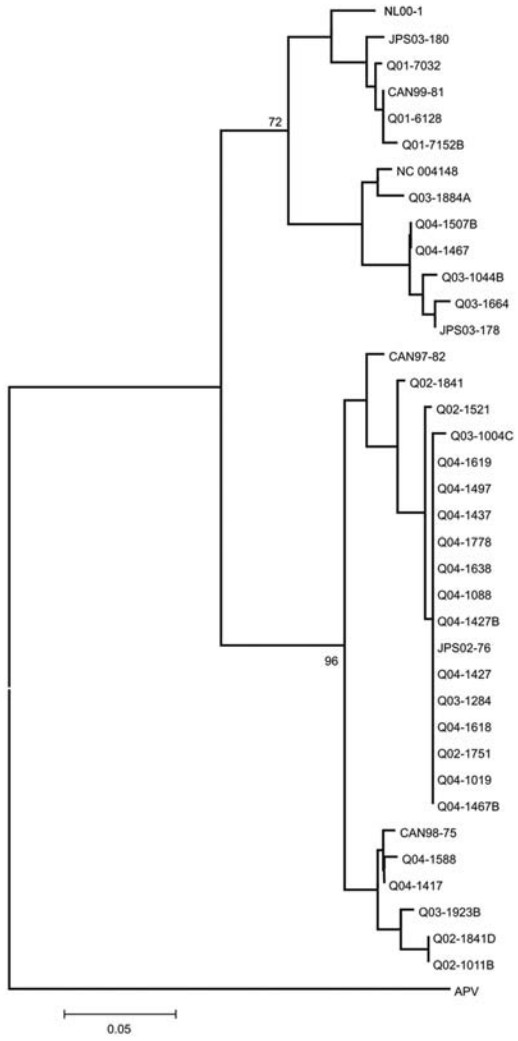

Figure 2. Phylogenetic analysis of the 182-nucleotide fragment of the phosphoprotein gene fragment of human metapneumovirus detected in respiratory samples collected in Australia. Sequences of avian pneumovirus (APV) type C (GenBank accession nos. AF176590 and AF176591) were used as outgroups to root the tree. Nucleotide sequences were aligned by using BioEdit version 7.0.0 and were subjected to neighbor-joining analysis with MEGA version 3.0 with 500 random bootstraps. CAN and NC, Canada; JPS, Japan; NL00–1, the Netherlands; Q, Queensland, Australia.

Table 2. Signs and symptoms noted with human metapneumovirus infection (N = 273)

Clinical feature	%
Cough	63
Rhinorrhea	61
Crackles/crepitations	60
Fever	57
Respiratory distress	48
Anorexia	45
Vomiting	39
Wheezing	38
Irritability	31
Tachypnea	30
Lethargy	26
Pharyngitis/tonsillitis	24
Dry mouth	23
Diarrhea	18
Otitis media	15
Noisy breathing	14
Rash	10
Conjunctivitis	7
Cyanosis	4
Apnea	2
Hoarseness	1

proportions, we saw no significant difference between patients with a co-infection and those without. Therefore, our data did not support the suggestion by others that co-infection of HMPV with RSV or other viral respiratory pathogens is a risk factor for severe disease (*6*).

The shift in predominant HMPV genotype observed in this study was similar to those reported for RSV and influenza viruses (*6*) and can be attributed to changes in immunity of the population in response to antigenic differences between the predominant circulating strains (*12*,*13*). However, a relationship between genotype and disease severity, as previously established for RSV (*14*,*15*), did not appear to apply for HMPV, but we plan to examine this relationship further.

The clinical features associated with HMPV infection in this study were not sufficiently distinctive to clinically differentiate it from other respiratory viral infections in children, particularly those attributed to RSV. In addition, few patients (10.6%) had severe disease, but most (76%) were sufficiently ill to be admitted and treated in the hospital for ≥3 days, which represents a substantial amount in healthcare costs.

Finally, this comprehensive study, conducted for 48 months, is the first one aimed at establishing an accurate estimate of the incidence and seasonal distribution of HMPV infection and to determine the genetic variation of HMPV circulating in our population. The clinical spectrum of infection in a substantial proportion of HMPV-positive patients has been described, and studies are continuing to fully elucidate the clinical effect of infection with this virus in our community.

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References

1. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med. 2001;7:719–24.
2. Nissen MD, Siebert DJ, Mackay IM, Sloots TP, Withers SJ. Evidence of human metapneumovirus in Australian children. Med J Aust. 2002;176:188.
3. Bastien N, Ward D, van Caeseele P, Brandt K, Lee SH, McNabb G, et al. Human metapneumovirus infection in the Canadian population. J Clin Microbiol. 2003;41:4642–6.
4. Freymouth F, Vabret A, Legrand L, Eterradossi N, Lafay-Delaire F, Brouard J, et al. Presence of the new human metapneumovirus in French children with bronchiolitis. Pediatr Infect Dis J. 2003;22:92–4.
5. Peiris JS, Tang WH, Chan KH, Khong PL, Guan Y, Lau YL, et al. Children with respiratory disease associated with metapneumovirus in Hong Kong. Emerg Infect Dis. 2003;9:628–33.
6. Robinson JL, Lee BE, Bastien N, Li Y. Seasonality and clinical features of human metapneumovirus infection in children in northern Alberta. J Med Virol. 2005;76:98–105.
7. van den Hoogen BG, Bestebroer TM, Osterhaus AD, Fouchier RA. Analysis of the genomic sequence of a human metapneumovirus. Virology. 2002;295:119–32.
8. Boivin G, Mackay IM, Sloots TP, Madhi S, Freymuth F, Wolf D, et al. Global genetic diversity of human metapneumovirus fusion gene. Emerg Infect Dis. 2004;10:1154–7.
9. Mackay IM, Bialasiewicz S, Jacob KC, McQueen E, Arden KA, Nissen MD, et al. Genetic diversity of human metapneumovirus over 4 consecutive years in Australia. J Infect Dis. 2006;193:160–3.
10. Syrmis MW, Whiley DM, Thomas M, Mackay IM, Williamson J, Siebert DJ, et al. A sensitive, specific, and cost-effective multiplex reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. J Mol Diagn. 2004;6:125–31.
11. Mackay IM, Bialasiewicz S, Waliuzzaman Z, Chidlow GR, Fegredo DC, Laingam S, et al. Genotyping of the human metapneumovirus using the P gene identifies four viral subtypes. J Infect Dis. 2004;190:1913–8.
12. Peret TC, Hall CB, Schnabel KC, Golub JA, Anderson LJ. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J Gen Virol. 1998;79:2221–9.

13. Cane PA, Pringle CR. Evolution of subgroup A respiratory syncytial virus: evidence for progressive accumulation of amino acid changes in the attachment protein. J Virol. 1995;69:2918–25.
14. Kneyber MC, Brandenburg AH, Rothbarth PH, de Groot R, Ott A, van Steensel-Moll HA. Relationship between clinical severity of respiratory syncytial virus infection and subtype. Arch Dis Child. 1996;75:137–40.
15. Walsh EE, McConnochie KM, Long CE, Hall CB. Severity of respiratory syncytial virus infection is related to virus strain. J Infect Dis. 1997;175:814–20.

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Community-acquired Methicillin-resistant *Staphylococcus aureus* in Children, Taiwan

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Highly virulent community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) with Panton-Valentine leukocidin (PVL) is common worldwide. Using antimicrobial drug susceptibility testing, staphylococcal cassette chromosome *mec* typing, exotoxin profiling, and pulsed-field gel electrophoresis typing, we provide evidence that supports the relationship between nasal strains of PVL-positive MRSA and community-acquired disease.

Panton-Valentine leukocidin (PVL) is a 2-component cytotoxin that targets human and rabbit polymorphonuclear cells, monocytes, and macrophages (*1*). Gene products of PVL (lukS-PV and lukF-PV), which are encoded by contiguously located, cotranscribed genes (*lukS-PV* and *lukF-PV*), assemble as hetero-oligomers and synergistically exert cytolytic pore-forming activity (*1*). PVL is mainly associated with primary cutaneous infections, especially furuncles, and with severe necrotizing community-acquired pneumonia (*2*). The PVL locus is present in most community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates studied and is a stable marker of CA-MRSA strains worldwide (*3*).

In a previous study, we found that CA-MRSA skin and soft tissue infections among Taiwanese children are caused by a predominantly endemic strain that has PVL genes (*4*). Nasal carriage of MRSA also plays a key role in the epidemiology and pathogenesis of community-associated disease (*5*). Therefore, our prospective investigation sought to 1) determine the prevalence of PVL-positive *S. aureus* among isolates from children with various staphylococcal diseases and asymptomatic nasal colonization, and 2) test the hypothesis that CA-MRSA infection is associated with community PVL-positive MRSA nasal carriage.

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The Study

The study protocol was reviewed and approved by the National Defense Medical Center Institutional Review Board. Two collections of *S. aureus* isolates were used. A list of all children ≤14 years of age hospitalized with various staphylococcal infections during the period from December 2003 to November 2005 was compiled from records at the clinical microbiology laboratory at the Tri-Service General Hospital in Taipei. The first collection of 144 infecting strains was further categorized into 11 types of staphylococcal infection on the basis of the clinical details provided. A case was considered community acquired if MRSA was isolated from cultures of specimens obtained within 72 hours after hospitalization. Risk factors for MRSA infection included hospitalization ≤12 months before the date of MRSA isolation; history of any surgical procedure; history of endotracheal intubation; underlying chronic disorder; antimicrobial drug therapy ≤12 months before the date of MRSA isolation; presence of an indwelling venous or urinary catheter; or household contact with a person with an identified risk factor or a worker in a healthcare environment (*6*).

A second collection of 300 colonizing strains was obtained during the same period by culturing samples from the anterior nares of 1,195 healthy children in the community. Eligible participants were ≤14 years of age with no acute medical problem who either visited a healthcare facility for a well-child checkup or attended 1 of 7 kindergartens in Taipei.

MRSA identification and antimicrobial drug susceptibility were determined according to the Clinical Laboratory Standards Institute (formerly known as the National Committee for Clinical Laboratory Standards) guidelines (*7*,*8*). Staphylococcal cassette chromosome *mec* (SCC*mec*) elements were typed and PVL genes were detected as described (*2*,*9*,*10*). Sequences specific for *sea* to *see*, *seg* to *sei*, *eta*, *etb*, and *tst*, which encode staphylococcal enterotoxins (SEA to SEE, and SEG to SEI), exfoliative toxins (ETA and ETB), and toxic shock syndrome toxin-1, respectively, were detected by using methods described by Jarraud et al. (*11*). Pulsed-field gel electrophoresis (PFGE) was performed by using a CHEF Mapper XA system (Bio-Rad Laboratories, Hercules, CA, USA) according to a published protocol (*12*).

Data were analyzed by the Mantel-Haenszel test and χ^2 test with SPSS version 10.0 software (SPSS, Chicago, IL, USA). A p value <0.05 was considered significant.

Of the 444 isolates examined, PVL-positive isolates constituted 23% of all *S. aureus* isolates analyzed (Table 1). Among 144 isolates (67 methicillin-susceptible *S. aureus* isolates and 77 MRSA isolates) from different staphylococcal infections, 82 (56.9%) were PVL positive, and most were associated with skin and soft tissue

Table 1. Association of Panton-Valentine leukocidin-positive *Staphylococcus aureus* isolates with types of staphylococcal infection and colonization*

Origin of sample	No. isolates	No. (%) PVL-positive isolates	Risk ratio (95% CI)†	p value‡
Furuncles	7	7 (100)	8.000 (1.279–50.040)	0.001
Abscess	9	8 (89)	7.111 (1.121–45.129)	0.002
Carbuncle	26	20 (77)	6.154 (0.972–38.959)	0.001
Cellulitis	25	19 (76)	6.080 (0.959–38.535)	0.002
Staphylococcal scarlet fever	27	17 (63)	5.037 (0.787–32.229)	0.013
Wounds§	20	5 (25)	2.000 (0.275–14.548)	0.475
Pyoderma	5	1 (20)	1.600 (0.127–20.219)	0.726
Pneumonia¶	8	1 (13)		
Bullous impetigo	6	0	NA/NM	NA/NM
Bacteremia	7	0	NA/NM	NA/NM
Other invasive infection#	4	4 (100)	8.000 (1.279–50.040)	0.006
Colonization	300	18 (6)	0.480 (0.073–3.169)	0.452
Total	444	100 (23)		

*PVL, Panton-Valentine leukocidin; CI, confidence interval; NA, not applicable; NM, not measured.
†Risk ratio is the ratio of the risk of being PVL positive in the presence of a particular type of infection or colonization to the absence of that type of infection or colonization.
‡By Mantel-Haenszel test.
§Mostly postsurgical.
¶Reference group for statistical analysis.
#Includes pyomyositis, osteomyelitis, and septic arthritis.

infections (especially furuncles). In contrast, only 18 (6%) of 300 colonizing isolates had the PVL locus. PVL genes were also found in isolates associated with other deep-space infections including pyomyositis, osteomyelitis, and septic arthritis. Of 1,195 healthy children who were screened, 89 (7.4%) had cultures with MRSA. Only 15 (16.9%) of 89 community MRSA-colonizing strains were PVL positive.

Among the 144 *S. aureus* isolates obtained from various types of staphylococcal disease, 32 (22.2%) isolates were further confirmed as CA-MRSA according to the inclusion criteria (abscess [n = 5], carbuncles [n = 6], cellulitis [n = 9], furuncles [n = 3], pyomyositis [n = 1], osteomyelitis [n = 1], pneumonia [n = 1], and staphylococcal scarlet fever [n = 6]); all contained the genes encoding PVL. Antibiograms of 15 PVL-positive MRSA-colonizing strains did not differ significantly from those of 32 CA-MRSA–infecting strains with respect to clindamycin, erythromycin, gentamicin, and chloramphenicol. All 47 PVL-positive MRSA isolates were resistant to penicillin G, and none had reduced susceptibility to vancomycin, teicoplanin, trimethoprim-sulfamethoxazole, fusidic acid, or ciprofloxacin.

To gain insight into the association between nasal strains of PVL-positive MRSA and community-acquired disease, we designed a comparative study in which the colonization and clinical samples were collected during the same period. Results of SCC*mec* typing and exotoxin profiling for both community MRSA-colonizing strains and CA-MRSA–infecting strains are shown in Table 2. Of 74 colonizing strains that did not have the PVL locus, most (94.6%) had SCC*mec* IV, and only 1 (1.35%) had SCC*mec* V_T. Conversely, irrespective of origin, PVL-positive MRSA strains were more likely to have SCC*mec* V_T than were the PVL-negative MRSA-colonizing strains ($p<0.001$). Regarding the exotoxin profiles, the most frequently encoded toxin gene among PVL-positive MRSA isolates was *seb* (97.9%). PVL-positive MRSA-colonizing strains and CA-MRSA–infecting strains were more likely to have genes that encoded SEB than were PVL-negative CA-MRSA–colonizing strains ($p = 0.006$). Genes for SEG/SEI were found only in PVL-negative MRSA-colonizing strains ($p<0.001$).

Diverse pulsotypes were found among the 47 PVL-positive MRSA strains subjected to PFGE typing (Figure). Two clusters that included 33 (70.2%) isolates were distinguished at the 70% similarity level. None of the isolates were linked on review of epidemiologic data derived from medical records. Except for 1 colonizing isolate (C7) and 2 infecting isolates (I22 and I23) that carried SCC*mec* IV, the other 30 (90.9%) isolates from clusters I and II had SCC*mec* V_T.

Conclusions

This is the first epidemiologic study of PVL-positive *S. aureus* in Taiwan. The prevalence of PVL-positive *S. aureus* among isolates collected from various types of staphylococcal infections was 56.9%; previous surveys reported rates of <5% to 12.4% (*13–15*). This higher prevalence is probably related to the greater proportion of pediatric patients with cutaneous infections. The frequencies of infections associated with these organisms were similar to those in previous studies (*2,13,14*). Overall, the outcomes of infections with PVL-positive strains were

Table 2. Distribution of staphylococcal cassette chromosome (SCC)*mec* types and exotoxin patterns among methicillin-resistant *Staphylococcus aureus* (MRSA) strains collected from community-acquired (CA) MRSA infections and nares cultures*

Characteristic	CA-MRSA–colonizing strains		CA-MRSA–infecting strains†
	PVL positive (n = 15)	PVL negative (n = 74)	(n = 32)
SCC*mec* type, no. (%) of isolates			
II	0	1 (1.35)	0
III	0	1 (1.35)	0
IIIA	0	1 (1.35)	0
IV	7 (46.7)	70 (94.6)‡	5 (15.6)
V_T§	8 (53.3)	1 (1.35)‡	27 (84.4)
No. (%) of isolates positive for production of other toxins			
ETA	0	2 (2.7)	0
ETB	1 (6.7)	2 (2.7)	0
TSST-1	0	5 (6.6)	1 (3.1)
SEA	1 (6.7)	6 (8.1)	0
SEB	14 (93.3)	57 (77.0)¶	32 (100)
SEC	0	4 (5.4)	0
SED	0	0	0
SEE	0	1 (1.4)	0
SEG/SEI	0	30 (40.5)‡	0
SEH	0	1 (1.4)	0

*PVL, Panton-Valentine leukocidin; ETA, exfoliative toxin A; ETB, exfoliative toxin B; TSST-1, toxic shock syndrome toxin-1; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEC, staphylococcal enterotoxin C; SED, staphylococcal enterotoxin D; SEE, staphylococcal enterotoxin E; SEG, staphylococcal enterotoxin G; SEI, staphylococcal enterotoxin I; SEH, staphylococcal enterotoxin H.
†All 32 CA-MRSA–infecting strains were PVL positive.
‡$p<0.001$ by χ^2 test for PVL-positive MRSA-colonizing strains and CA-MRSA–infecting strains vs. PVL-negative MRSA-colonizing strains.
§V_T refers to the SCC*mec* V_T element in strain TSGH 17 from Taiwan (*10*).
¶$p = 0.006$ by χ^2 test for PVL-positive MRSA-colonizing strains and CA-MRSA–infecting strains vs. PVL-negative MRSA-colonizing strains.

excellent and comparable to that of PVL-negative strains. Only 1 death occurred.

Four factors that support the relationship between nasal strains of PVL-positive MRSA and community-acquired disease. First, both PVL-positive MRSA-colonizing strains and CA-MRSA–infecting strains had consistent antibiograms. Second, most PVL-positive MRSA-colonizing strains and CA-MRSA–infecting strains had SCC*mec* V_T, whereas a high prevalence of SCC*mec* IV was found among PVL-negative MRSA-colonizing strains. Third, PVL-positive MRSA-colonizing strains and CA-MRSA–infecting strains had consistent exotoxin profiles, which differed from those of PVL-negative MRSA-colonizing strains. Fourth, PFGE findings indicate that some

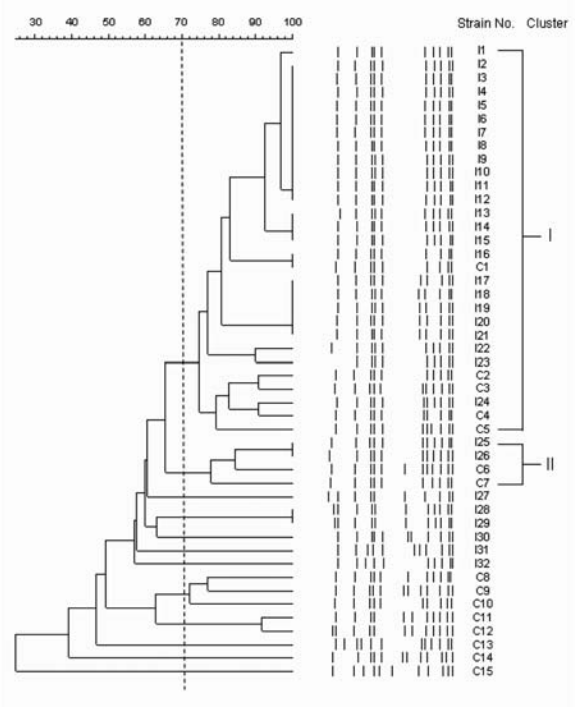

Figure. Pulsed-field gel electrophoresis patterns and phylogenetic tree of 47 methicillin-resistant *Staphylococcus aureus* (MRSA) isolates with Panton-Valentine leukocidin (PVL) genes. Banding patterns were digitalized and analyzed with Molecular Analyst Fingerprinting, Fingerprinting Plus, and Fingerprinting DST software (Bio-Rad Laboratories, Hercules, CA, USA). The grouping method was performed to deduce a dendrogram from the matrix by the unweighted pair group method with arithmetic averages clustering technique after calculation of similarities using the Pearson correlation coefficient between each pair of organisms. The scale indicates the level of pattern similarity. Similarities >70% represent clonal spread of strains. The first letter of each isolate designation indicates the type of the isolate as follows: I, community-acquired MRSA-infecting isolates; C, PVL-positive MRSA-colonizing isolates.

PVL-positive MRSA isolates that colonized children who remained asymptomatic were clonally related to clinically isolated CA-MRSA–infecting strains, especially those with SCC*mec* V_T, compared with SCC*mec* IV (6/8, 75% and 1/7, 14.3%, respectively).

Several study limitations merit consideration. First, our study is a snapshot in time because the molecular epidemiology of CA-MRSA is constantly changing. Second, we were unable to determine risk for infection because children colonized with community PVL-positive MRSA were not followed-up longitudinally. Finally, our results are geographically distinct and may not be generalized to the global population.

Our study showed that PVL genes are carried by a large number of *S. aureus* isolates, especially among those causing disease. We provide evidence that links community PVL-positive MRSA-colonizing strains to CA-MRSA–infecting strains from various types of staphylococcal infection.

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References

1. Prevost G, Mourey L, Colin DA, Menestrina G. Staphylococcal pore-forming toxins. Curr Top Microbiol Immunol. 2001;257:53–83.
2. Lina G, Piémont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V, et al. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis. 1999;29:1128–32.
3. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis. 2003;9:978–84.
4. Wang CC, Lo WT, Chu ML, Siu LK. Epidemiological typing of community-acquired methicillin-resistant *Staphylococcus aureus* isolates from children in Taiwan. Clin Infect Dis. 2004;39:481–7.
5. Pan ES, Diep BA, Charlebois ED, Auerswald C, Carleton HA, Sensabaugh GF, et al. Population dynamics of nasal strains of methicillin-resistant *Staphylococcus aureus* and their relation to community-associated disease activity. J Infect Dis. 2005;192:811–8.
6. Hussain FM, Boyle-Vavra S, Bethel CD, Daum RS. Current trends in community-acquired methicillin-resistant *Staphylococcus aureus* at a tertiary care pediatric facility. Pediatr Infect Dis J. 2000;19:1163–6.
7. National Committee for Clinical Laboratory Standards. Methods for disk diffusion: approved standard M2–A8: performance standards for antimicrobial disk susceptibility tests. Wayne (PA): The Committee; 2003.
8. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing: 14th informational supplement. NCCLS document M100–S14. Wayne (PA): The Committee; 2004.
9. Oliveira DC, de Lencastre H. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 2002;46:2155–61.
10. 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.
11. Jarraud S, Cozon G, Vandenesch F, Bes M, Etienne J, Lina G. Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. J Clin Microbiol. 1999;37:2446–9.
12. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol. 2003;41:5113–20.
13. Prevost G, Couppie P, Prevost P, Gayet S, Petiau P, Cribier B, et al. Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. J Med Microbiol. 1995;42:237–45.
14. Holmes A, Ganner M, McGuane S, Pitt TL, Cookson BD. Kearns AM. *Staphylococcus aureus* isolates carrying Panton-Valentine leukocidin genes in England and Wales: frequency, characterization, and association with clinical disease. J Clin Microbiol. 2005;43:2384–90.
15. Yamasaki O, Kaneko J, Morizane S, Akiyama H, Arata J, Narita S, et al. The association between *Staphylococcus aureus* strains carrying Panton-Valentine leukocidin genes and the development of deep-seated follicular infection. Clin Infect Dis. 2005;40:381–5.

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Incubation Period of Hantavirus Cardiopulmonary Syndrome

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The potential incubation period from exposure to onset of symptoms was 7–39 days (median 18 days) in 20 patients with a defined period of exposure to Andes virus in a high-risk area. This period was 14–32 days (median 18 days) in 11 patients with exposure for ≤48 hours.

Hantaviruses are RNA viruses that are harbored by specific rodent species and transmitted to humans by inhalation of virus-contaminated rodent feces, urine, and saliva (*1*). Human hantavirus syndromes include hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) (*2*). The latter is also known as hantavirus pulmonary syndrome, but we prefer HCPS because most deaths result from cardiogenic shock (*3*,*4*).

Although HCPS is a serious problem in North and Central America, more cases of HCPS and deaths from this disease occur in South America; in Chile, 469 cases have been reported through March 2, 2006, with a case-fatality rate of 36% (*5*,*6*). Both Sin Nombre virus (SNV), the primary cause of HCPS in North America, and Andes virus, the cause of HCPS in Chile and most cases in Argentina, cause a severe form of HCPS. However, Andes virus is unique among hantaviruses in that it can be transmitted from person to person (*7*).

Human contact with *Oligoryzomys longicaudatus* (rice rat or colilargo), the reservoir of Andes virus, occurs in rural areas in central and southern Chile (from 28°S to 51°S). In Chile, 70% of the patients have a history of occupational or peridomestic exposure to rodents or peridomestic exposure to a human with HCPS; in 20% to 35%, exposure is limited to visiting high-risk areas for recreational purposes (*8*).

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The incubation period for HCPS caused by Andes virus has not been reported. The incubation period for HCPS caused by SNV has been reported to be 9–33 days (*9*). The incubation period for HFRS has been estimated to be 1–6 weeks (*10*,*11*) but was reported as 11–23 days after intramuscular or intravenous challenge in volunteers (*12*).

The Study

To define the incubation period for Andes virus infection, we identified 20 patients with a well-defined period of exposure to a high-risk area among 106 persons with HCPS enrolled in research protocols (treatment interventions, contact studies, quantitative viremia during HCPS) or interviewed by 1 of the authors. Nineteen of 20 were residents of Santiago or other urban areas who traveled to a high-risk area for recreational purposes. In each case, the person resided in an urban area without Andes virus–infected rodents and rodent-to-human transmission and then traveled for a defined period to a high-risk area where rodent-to-human transmission has occurred and where Andes virus–infected rodents were found (*13*). Nineteen patients reported a variety of risky activities, such as entering or cleaning previously unused cabins or houses, camping, or clearing land. The other patient (no. 11) was a biologist who was bitten on the finger by a rodent, which he identified as *O. longicaudatus* that he had trapped in a rural area.

The exposure period was defined as the number of days from arrival to departure in a high-risk area. The maximum incubation period was the time from arrival at the high-risk area to the onset of symptoms, and the minimum incubation was the time from departure from the high-risk area to onset of symptoms. The prodrome was defined as the period from the onset of fever or other constitutional symptoms until the onset of the cardiopulmonary phase and hospitalization.

Confirmation of HCPS was based on the clinical syndrome with laboratory confirmation by ≥1 of the following tests: ELISA for immunoglobulin G (IgG) and IgM antibody for hantavirus, a focus reduction assay for neutralizing antibody to Andes virus, and an RNA reverse transcription (RT)-PCR for Andes virus. Laboratory confirmation was by IgG and IgM ELISA in 8 patients; IgG and IgM ELISA plus Andes virus neutralizing antibody in 3 patients; IgG and IgM ELISA, Andes virus neutralizing antibody, and RT-PCR in 7 patients; IgG and IgM ELISA plus RT-PCR in 1 patient; and RT-PCR in 1 patient. Patients had a mean age of 30.5 years (range 2–68 years); 65% were male. The clinical course was characterized as severe (respiratory failure and shock) in 14, moderate (respiratory failure without shock) in 1, and mild (respiratory failure without requiring mechanical ventilation) in 5. Four of 20 patients died.

The potential maximum incubation period for all 20 patients was 11–39 days, and the potential minimum incubation period was 7–32 days. The median incubation period for all 20 patients was 18 days (range 7–39 days) (Figure). Among the 11 patients (nos. 10–20) with exposure ≤48 hours, the potential incubation period was 14–32 days (median 18 days). The incubation periods of patients with mild cases (range 12–34 days) did not differ from those of patients with moderate and severe cases (7–39 days).

Conclusions

Our study is the first to determine the incubation period for Andes virus infection. It provides the most complete evaluation of the incubation period for rodent-to-human transmission for the New World hantaviruses, including Andes virus and SNV. Young et al. reported 11 patients with SNV infection with well-defined exposure to rodents (*9*). However, only maximum or minimum incubation periods could be determined for 4 patients. In the 7 patients for whom both minimum and maximum incubation periods could be calculated, the incubation period had a range of 9 to 33 days. St Jeor reported SNV infection in 2 children hospitalized 3 weeks after they were bitten by a mouse (*14*), but the time between the bite and the onset of symptoms was not reported.

Human-to-human transmission of Andes virus infection has been reported in Argentina (*7*,*15*) and Chile where human-to-human transmission is strongly suggested in household clusters of HCPS cases (M. Ferrés, pers. comm.). In the 1996 outbreak in Argentina, both epidemiologic and molecular evidence supported person-to-person transmission (*7*,*15*). The time between disease onset in 14 cases of person-to-person transmission among 16 patients with HCPS was 4–28 days. However, these intervals should be interpreted with caution. They are based on the mode of transmission considered most likely by Wells et al. (*15*), but there were multiple cases in which patients had contact with >1 potential source patient. Furthermore, these were intervals between onset of symptoms in the proposed source and in subsequent patients, and with 4 exceptions, were not calculations of an incubation period based on defined periods of exposure to the proposed source patient. The duration of exposure to source patients was reported for only 4 case-patients, including 3 occupants of a car in whom symptoms developed at 11, 15, and 29 days, respectively, after a daylong car trip with an index patient who was symptomatic. The shortest interval of 4 days was for a patient who had close contact with another patient 10 days before symptoms developed. If this patient is considered to be a more likely source, as it was by Wells et al., the range would be 10–28 days.

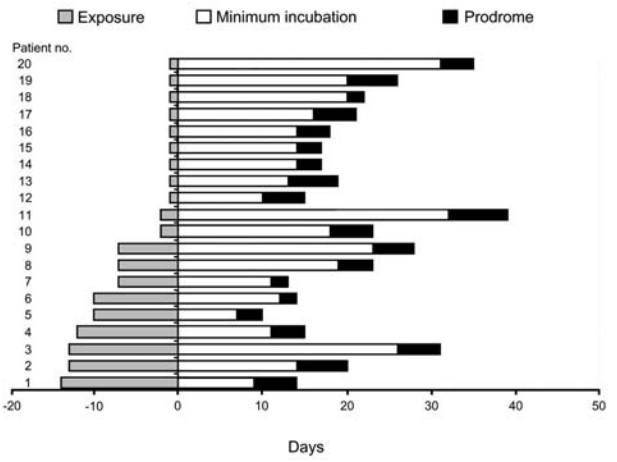

Figure. Incubation period for 20 patients in Chile in whom hantavirus cardiopulmonary syndrome caused by Andes virus developed after various periods of exposure. All patients progressed to the cardiopulmonary phase and were hospitalized at the end of the prodrome.

In summary, our data for 11 patients in whom exposure was limited to ≤48 hours showed a potential incubation period of 14 to 32 days and a median of 18 days. Inclusion of patients with exposure periods ≤14 days provided a potential incubation period of 7 to 39 days. These data provide the most complete evaluation of the incubation period for HCPS caused by Andes or SNV and are consistent with available data for the incubation period for HFRS (*7*,*9*–*12*,*14*,*15*).

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References

1. Botten J, Mirowsky K, Ye C, Gottlieb K, Saavedra M, Ponce L, et al. Shedding and intracage transmission of Sin Nombre hantavirus in the deer mouse (*Peromyscus maniculatus*) model. J Virol. 2002;76:7587–94.
2. Schmaljohn C, Hjelle B. Hantaviruses: a global disease problem. Emerg Infect Dis. 1997;3:95–104.
3. Mertz GJ, Miedzinski L, Goade D, Pavia AT, Hjelle B, Hansbarger CO, et al. Placebo-controlled, double-blind trial of intravenous ribavirin for hantavirus cardiopulmonary syndrome in North America. Clin Infect Dis. 2004;39:1307–13.
4. Hallin GW, Simpson SQ, Crowell RE, James DS, Koster FT, Mertz GJ, et al. Cardiopulmonary manifestations of the hantavirus pulmonary syndrome. Crit Care Med. 1996;24:252–8.

5. Duchin JS, Koster F, Peters CJ, Simpson GL, Tempest B, Zaki R, et al. Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. N Engl J Med. 1994;330:949–55.
6. Pini N. Hantavirus pulmonary syndrome in Latin America. Curr Opin Infect Dis. 2004;17:427–31.
7. Padula PJ, Edelstein A, Miguel SD, Lopez NM, Rossi CM, Rabinovich RD. Hantavirus pulmonary syndrome outbreak in Argentina: molecular evidence for person-to-person transmission of Andes virus. Virology. 1998;241:323–30.
8. Sotomayor V, Aguilera X. Epidemiología de la infección humana por hantavirus en Chile. Revista Chilena de Infectologia. 2000;17:220–32.
9. Young JC, Hansen GR, Graves TK, Deasy MP, Humphreys JG, Fritz CL, et al. The incubation period of hantavirus pulmonary syndrome. Am J Trop Med Hyg. 2000;62:714–7.
10. Kulagin CM, Fedorova H, Ketiladze EC. Laboratory outbreak of hemorrhagic fever with renal syndrome (clinico-epidemiological characteristics). Journal of Microbiology, Epidemiology and Immunology. 1962;33:121–6.
11. Powell GM. Hemorrhagic fever: a study of 300 cases. Medicine (Baltimore). 1954;33:97–153.
12. Smorodintsev AA. Etiologiaa gemorracheskogo nefrozo-nefrita. Moscow: Medgiz; 1944. p. 28–38.
13. Torres-Pérez F, Navarrete-Droguett J, Aldunate R, Yates TL, Mertz GJ, Vial PA, et al. Peridomestic small mammals associated with confirmed cases of human hantavirus disease in southcentral Chile. Am J Trop Med Hyg. 2004;70:305–9.
14. St Jeor SC. Three-week incubation period for hantavirus infection. Pediatr Infect Dis J. 2004;23:974–5.
15. Wells RM, Sosa Estani S, Yadon ZE, Enria D, Padula P, Pini N, et al. An unusual hantavirus outbreak in southern Argentina: person-to-person transmission? Emerg Infect Dis. 1997;3:171–4.

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Bat-associated Rabies Virus in Skunks

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Rabies was undetected in terrestrial wildlife of northern Arizona until 2001, when rabies was diagnosed in 19 rabid skunks in Flagstaff. Laboratory analyses showed causative rabies viruses associated with bats, which indicated cross-species transmission of unprecedented magnitude. Public health infrastructure must be maintained to address emerging zoonotic diseases.

In North America, >90% of cases of rabies in animals occur in wildlife (*1*); several mammalian taxa harbor characteristic rabies virus variants (RABVV). In Arizona, skunks (*Mephitis mephitis*) and gray foxes (*Urocyon cinereoargenteus*) maintain independent rabies enzootic cycles, and in indigenous bats, rabies has been diagnosed in 14 of 28 species (Arizona Department of Health Services, unpub. data). Although skunks live throughout Arizona, until 2001, rabid skunks had been found only in the southeastern quadrant of the state.

In the United States, bat RABVV are a source of infection for humans and other mammals (*2–8*). Typically, interspecies infection produces a single fatal spillover event; secondary transmission has rarely been observed. Antigenic typing of rabid carnivores in Arizona from 1996 through 2000 identified bat RABVV in 1 domestic dog and 2 gray foxes. This report describes the largest documented rabies epizootic among terrestrial mammals infected with bat RABVV, with perpetuated animal-to-animal transmission. Coincident with the zoonotic disease significance, this report provides contemporary insight into pathogen evolution (*9*).

The Study

In January 2001, a homeowner contacted Flagstaff Animal Control about a dead skunk. Although no human had been exposed to the skunk, tissues were submitted to the Arizona State Health Laboratory, where rabies was diagnosed. This skunk was the first rabid terrestrial wild carnivore reported from the area. The Texas Department of State Health Services subsequently identified an RABVV associated with bats in tissues sent for antigenic characterization. From January through April, 14 more skunks, dead or exhibiting abnormal behavior, were found throughout a large residential subdivision within 4 km of the initial case. All were infected with the same bat RABVV. From April through July, 4 more skunks infected with bat RABVV were identified ≈9 km west of the initial focus (Figure 1). Control measures included prohibiting relocation of nuisance skunks, comprehensive public education, pet rabies vaccine clinics, and a 90-day emergency quarantine requiring pets to be leashed or confined and vaccinated (Figure 1). Additionally, 217 urban skunks were vaccinated and marked with ear tags during a 6-month phased program of trap, vaccinate, and release.

In Flagstaff and the surrounding county, during the decade before this epizootic, 2 rabid bats, on average, were reported each year. During the epizootic, 218 animals were submitted for rabies testing (Table). Rabies was confirmed in 19 (13%) of 145 tested skunks and 2 (9%) of

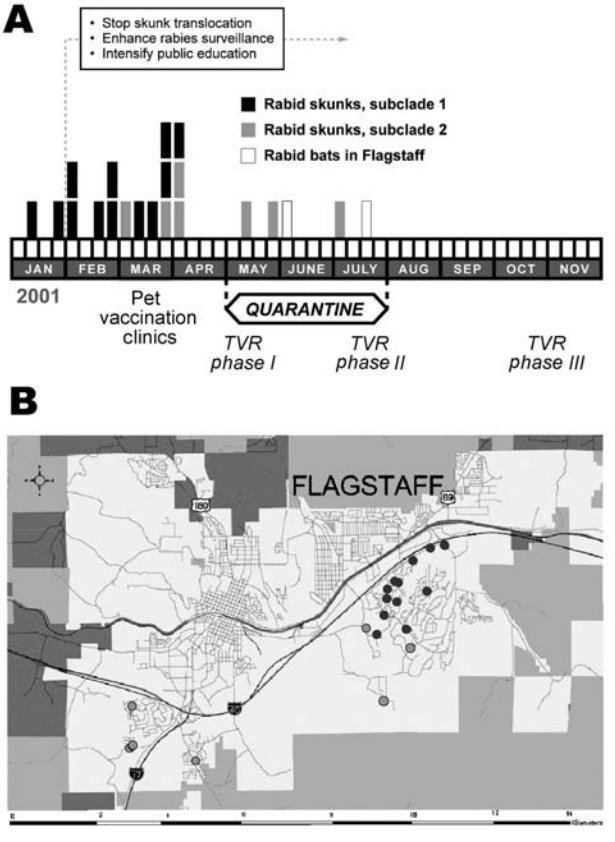

Figure 1. Temporal and geographic distribution of rabies outbreak in Flagstaff, Arizona. A) Timeline and control measures. TVR: trap, vaccinate, release program. B) Geographic location of rabid skunks (dark gray dots = subclade 1, light gray dots = subclade 2).

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Table. Animals from Flagstaff submitted for rabies diagnosis, January–July, 2001

Animal	Scientific name	No. submitted	No. rabid
Skunk	*Mephitis mephitis*	145	19
Bat	(Multiple spp.)	22	2
Domestic cat	*Felis domesticus*	12	0
Gray fox	*Urocyon cinereoargenteus*	9	0
Domestic dog	*Canis familiaris*	9	0
Squirrel	Species unknown	8	0
Coyote	*Canis latrans*	4	0
Raccoon	*Procyon lotor*	2	0
Porcupine	*Erethizon dorsatum*	2	0
Prairie dog	*Cynomys ludovicianus*	2	0
Badger	*Taxidea taxus*	1	0
Opossum	*Didelphis virginiana*	1	0
Bobcat	*Lynx rufus*	1	0
Total		218	21

22 tested bats. Although most (18 [95%]) of the rabid skunks were identified and reported by lay citizens, no contact between these skunks and humans or domestic animals was reported.

Local baseline population estimates were not available to indicate whether skunk demography affected disease attributes. Synchronous with this outbreak, independent epizootic activity caused by well-established skunk RABVV was documented in southern Arizona, which suggests that regional skunk epizootiologic dynamics were similarly affected. Skunks' seasonal behavior may have contributed to transmission events. This epizootic was initially recognized when a dead skunk appeared in a snow-covered backyard, during a season when skunks are in communal dens. Given an incubation period of 2 months, most transmission would have occurred between late autumn (when skunks are in their dens) and late winter (when they are mating).The Flagstaff epizootic peak coincided with nationwide seasonal trends of rabid skunks (*1*). Enhanced postepizootic surveillance in Flagstaff did not detect additional rabid terrestrial mammals for the next 3 years. However, in 2004, a total of 5 skunks found in the initially affected east Flagstaff neighborhood and 1 fox 28 km south of Flagstaff were infected with the same bat RABVV (*10*).

Viruses isolated from the rabid skunks exhibited monoclonal antibody patterns similar to RABVV associated with big brown (*Eptesicus fuscus*) and *Myotis* bats in the western United States (*11*). These are among the most abundant bat species in Arizona and often roost in houses and outbuildings; however, no bat colonies were found in association with any of the rabid skunks. Restriction digests of PCR amplicons from the rabid skunks did not match patterns known for RABVV from North American terrestrial reservoirs (*12*). Phylogenetic analysis of a 300-bp region of the N gene showed that the Flagstaff skunk RABVV was identical (100%) to Arizona bat RABVV (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no08/05-1526-appT.htm,and Figure 2A), and differed by 22% from skunk and gray fox RABVV. A monophyletic clade (clade A) of 8/8 big brown, 5/14 *Myotis*, and 1/6 southern yellow (*Lasiurus ega*) bats shared >95% identity with Flagstaff skunk RABVV. An additional 44 samples, representing 11 bat species, differed by >8% from Flagstaff skunk RABVV.

An analysis of clade A, which incorporates N and G genes, indicated that the Flagstaff skunk RABVV were more closely related to 2 bat RABVV (*E. fuscus* from Coconino County, *M. velifer* from Maricopa County) collected in 1999 and 1997 than to the 2 bat RABVV collected locally during the outbreak. In clade B, subclade 1 RABVV were collected from January through early April

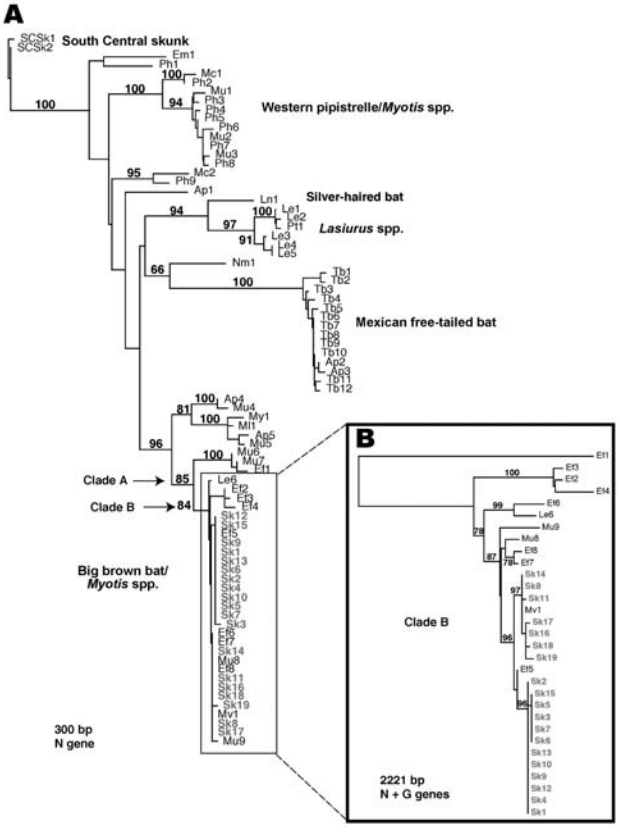

Figure 2. A) Phylogenetic tree of the 19 rabid skunk isolates and representative samples of known rabies virus variants (RABVV) from Arizona based on 300 bp of the nucleoprotein (N) gene (GenBank accession no. AY170226-304). B) Detailed analyses of clade including all 19 skunk isolates (clade B) based on 2221 bp of the N and glycoprotein (G) genes (GenBank accession no. AY170397-438). Phylogenetic analyses used PAUP* software (version 4.0b2, Sinauer Associates, Sunderland, MA, USA; 2000] using the neighbor-joining search algorithm (minimum evolution) with maximum likelihood to estimate Ti:Tv ratio and nucleotide base frequencies (HKY85 model). Numbers at tree nodes indicate nonparametric bootstrap proportions based on 1,000 replicates.

from the northeastern region of the outbreak, whereas subclade 2 RABVV were collected from early March through July from the southeastern and western regions of the outbreak (Figure 1). However, phylogenetic data do not support a wavelike spread from northeast to west because this would require nesting of subclade 1 within subclade 2. In contrast, both subclades exhibit independently derived mutations. East-to-west epizootic movement of RABVV within subclade 2 (sk16–19 form a monophyletic clade nested within subclade 2) during April is supported by the data and may be related to dispersal of infected skunks along river corridors or translocation by humans. One person reported trapping, moving, and releasing a skunk before the outbreak was known in the community. Alternatively, apparent shifts may be an artifact of intensified public awareness and reporting. Lack of sampling in the uninhabited forest between the eastern and western foci limits our ability to discriminate among these hypotheses.

Conclusions

This is the largest recorded cluster of bat RABVV infection in terrestrial mammals. Investigation of this novel outbreak showed evolution in action with the emergence of an RABVV that successfully adapted from Chiroptera to Carnivora. Previously documented clusters involving 3–4 to terrestrial mammals infected with a single insectivorous bat rabies virus variant did not corroborate sustained transmission (*12*). Although >1 skunk may have been exposed to a single rabid bat, it is highly unlikely that each skunk was exposed to the same bat or that multiple bat-skunk exposures occurred. We could not ascertain the complete scope of this outbreak or whether it was the index event. Phylogenetic analyses support the evolution of 2 independent lineages, suggesting establishment for months or years. Additionally, virus isolation from salivary glands of 5 affected skunks and the reappearance of rabid skunks with the same RABVV in 2004 support the probability of independent transmission.

The recognition of this epizootic can be credited to a coordinated laboratory-based disease surveillance program to monitor sick and dead wildlife for potential zoonoses (plague, tularemia, rabies) even in situations lacking human or pet exposures. Comprehensive animal disease surveillance provides direct benefits to public health and animal health by promoting early recognition of risk and opportunities for disease control and prevention interventions.

Unpredictable health threats related to emerging zoonoses, especially those involving wildlife reservoirs, pose notable surveillance and control challenges (*13–15*). Recent bioterrorism initiatives emphasize integration of human and animal disease surveillance, and enhanced laboratory capacity, as essential functions in zoonosis detection (*13*). Rabies surveillance and control programs serve as historic prototypes for effective, long-term, public health programs. Quintessential zoonotic disease programs require innovative and expanded capacities, commitments to public health and veterinary laboratory infrastructure, and appropriate interagency and interdisciplinary coordination and communication.

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References

1. Krebs JW, Mandel EJ, Swerdlow D, Rupprecht CE. Rabies surveillance in the United States during 2003. J Am Vet Med Assoc. 2004;225:1837–49.
2. Brass DA. Rabies in bats: natural history and public health implications. Ridgefield (CT): Livia Press; 1994.
3. Messenger SL, Smith JS, Orciari LA, Yager PA, Rupprecht CE. Emerging patterns of rabies deaths and increased viral infectivity. Emerg Infect Dis. 2003;9:151–4.
4. Mondul AM, Krebs JW, Childs JE. Trends in national surveillance for rabies among bats in the United States (1993–2000). J Am Vet Med Assoc. 2003;222:633–9.
5. Anderson LJ, Nicholson MB, Tauxe RV, Winkler WG. Human rabies in the United States, 1960 to 1979: epidemiology, diagnosis, and prevention. Ann Intern Med. 1984;100:728–35.
6. Noah DL, Drenzek CL, Smith JS, Krebs JW, Orciari L, Shaddock J, et al. Epidemiology of human rabies in the United States, 1980–1996. Ann Intern Med. 1998;128:922–30.
7. Rohde RE, Mayes BC, Smith JS, Neill SU. Bat rabies, Texas, 1996–2000. Emerg Infect Dis. 2004;10:948–52.
8. McQuiston JH, Yager PA, Smith JS, Rupprecht CE. Epidemiologic characteristics of rabies virus variants in dogs and cats in the United States, 1999. J Am Vet Med Assoc. 2001;218:1939–42.
9. Badrane H, Tordo N. Host switching in Lyssavirus history from the Chiroptera to the Carnivora orders. J Virol. 2001;75:8096–104.
10. Arizona Department of Health Services. Rabies in Arizona, 2004. [cited 2006 Mar 26]. Available from http://www.azdhs.gov/phs/oids/vector/rabies/rab04.htm

11. Smith JS, Reid-Sanden FL, Roumillat LF, Trimarchi C, Clark K, Baer GM, et al. Demonstration of antigenic variation among rabies virus isolates by using monoclonal antibodies to nucleocapsid proteins. J Clin Microbiol. 1986;24:573–80.
12. Rohde RE, Neill SU, Clark KA, Smith JS. Molecular epidemiology of rabies epizootics in Texas. Clin Diagn Virol. 1997;8:209–17.
13. Messenger SL, Rupprecht CE, Smith JS. Bats, emerging virus infections, and the rabies paradigm. In: Kunz TH, Fenton MB, editors. Bat ecology. Chicago: The University of Chicago Press; 2003. p. 622–79.
14. Centers for Disease Control and Prevention. Core functions and capabilities of state public health laboratories: a report of the Association of Public Health Laboratories. MMWR Recomm Rep. 2002;51 (RR14):1–8.
15. Hanlon CA, Childs JE, Nettles VF. Recommendations of a national working group on prevention and control of rabies in the United States. Article III: rabies in wildlife. J Am Vet Med Assoc. 1999;215:1612–8.

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Fecal Viral Load and Norovirus-associated Gastroenteritis

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We report the median cDNA viral load of norovirus genogroup II is ≥100-fold higher than that of genogroup I in the fecal specimens of patients with norovirus-associated gastroenteritis. We speculate that increased cDNA viral load accounts for the higher transmissibility of genogroup II strains through the fecal-oral route.

Norovirus (NoV), a member of the family *Caliciviridae* in the genus *Norovirus*, is a major causative agent of viral gastroenteritis, affecting all age groups worldwide (*1*). NoVs are clustered into 5 genogroups; genogroup I (GI), GII, and GIV infect humans (*2*). Molecular epidemiologic studies in different countries and regions show that NoV GII is the predominant genogroup circulating in the community; it accounts for most sporadic, nosocomial, and outbreak cases (*3*). However, its predominance cannot be entirely explained. In this study, we show for the first time that the median cDNA viral load of NoV GII is ≥100-fold higher than that of GI in fecal specimens of patients with NoV-associated gastroenteritis. This finding suggests possible higher transmissibility of GII strains through the fecal-oral route.

The Study

From December 2004 through November 2005, a total of 651 fecal specimens were collected within 48 hours of symptom onset from 627 patients (43.5% male, <1–97 years of age, 26.9% <16 years of age) with symptoms of gastroenteritis at Prince of Wales Hospital, Hong Kong Special Administrative Region, People's Republic of China. All cases were sporadic (defined as having no known related cases). Fecal specimens were stored at –70°C after collection and were processed in batches monthly. Local monthly mean air temperature during the study period was obtained from the Hong Kong Observatory (available from http://www.hko.gov.hk/wxinfo/pastwx/ywx.htm).

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Viral RNA was purified from fecal specimens and transcribed to cDNA as described (*4*). All specimens had a detectable level of human β-actin cDNA, which suggests high RNA integrity. Filter tips were used throughout the study to minimize cross-contamination. NoV GI and GII were detected by a quantitative and genogroup-specific real-time PCR assay, as previously described (*5*). Sterile water was used in place of cDNA as negative control. Three amplicons from each genogroup were directly sequenced to confirm their identities and genogroups on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). cDNA viral load was quantified in triplicate per run against 10-fold serial dilutions (10^8–10^1 copies) of external plasmid standards prepared by cloning genogroup-specific amplicons into vector pCR2.1-TOPO (Invitrogen Corp., Carlsbad, CA, USA). The lower detection limit of the assay was equivalent to 2×10^4 copies of cDNA per gram of fecal specimen. Coefficients of variation within and between runs were calculated as the percentage of the ratio between the standard deviation and mean of threshold cycle numbers from the standard curves. The respective intra- and interassay coefficients of variation for NoV GI were 0%–4.1% and 1.9%–5.8%, respectively, and 0.1%–6.1% and 2.7%–6.6%, respectively, for NoV GII. These findings indicate the high reproducibility in viral load quantitation by the assay. Two common gastroenteritis-associated viruses, sapovirus and group A rotavirus, were detected in parallel, as previously described (*4*,*6*).

Phylogenetic analysis of NoV isolates was performed by using primer sets G1FF/G1SKR and G2FB/G2SKR for GI and GII, respectively, as described elsewhere (*7*). Isolates were clustered with the nomenclature system of Zheng et al. (*2*). Since G1FF/G1SKR and G2FB/G2SKR completely spanned the region amplified by the RT-PCR assay, the 2 primer sets were also used to check for sequence complementarity among target, primers, and probe used in quantitation. Statistical analyses were performed by SPSS version 11.5.1 (SPSS Inc., Chicago, IL, USA), and figures were constructed by Prism version 4.03 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS.

NoVs were detected in 54 (8.3%) fecal specimens. Among the NoV-positive specimens, 8 (14.8%) were infected with GI, 37 (68.5%) with GII, and 9 (16.7%) were coinfected with GI and GII. Moreover, 3 (5.6%) specimens were coinfected with sapovirus, 2 (3.7%) with group A rotavirus, and 1 (1.9%) with sapovirus and group A rotavirus. The mean age of patients infected with NoV GI and GII was 54.6 and 33.0 years, respectively ($p = 0.02$). Sex and hospitalization rates between patients infected with the 2 genogroups did not differ significantly (Table).

The median cDNA viral load of NoV GI and GII detected in the fecal specimens was 8.4×10^5 (range 2.2×10^4 –

Table. Characteristics of patients infected with norovirus (NoV) genogroup I (GI) and GII

Characteristics	GI (n = 8)	GII (n = 37)	GI/II coinfection (n = 9)	p value*
Male sex, no. (%)	3 (38)	21 (57)	4 (44)	0.44
Mean age, y (range)	54.6 (13–85)	33.0 (1–74)	42.2 (12–65)	0.02
Hospitalization, no. (%)	4 (50)	11 (30)	1 (11)	0.41

*NoV GI versus GII only.

2.9×10^{10}) and 3.0×10^{8} (range 2.5×10^{4}–7.7×10^{10}) copies per gram of fecal specimen (Figure 1A), respectively. Although the range was comparable between the genogroups, the median of NoV GII was ≥100-fold higher than that of GI (p = 0.0022, 2-tailed Mann-Whitney U test). Similar findings were obtained when NoV GI/GII coinfections (p = 0.0066, Figure 1B) or all viral coinfections (p = 0.0042, Figure 1C) were excluded. Seven of 9 specimens with NoV GI/GII coinfections had higher cDNA viral load of GII than GI, with fold changes from 4 to 452 (median 248) (Figure 2). Furthermore, while NoV was detected year-round, a marked seasonal trend was evident: higher prevalence occurred in winter months, when the cDNA viral load of GII was generally higher compared with GI (online Appendix Figure 1, available from http://www.cdc.gov/ncidod/EID/vol12no08/06-0081-appG1.htm).

Multivariate linear regression model was used to determine the potential association between cDNA viral load, NoV genogroup, and patient's age. After age stratification, the cDNA viral load in fecal specimen was still significantly associated with NoV genogroup (β = 0.390, p = 0.002). However, no significant association was found between cDNA viral load and age of patients (β = –0.060, p = 0.626).

Of the 63 NoV isolates, 43 (68.3%) were successfully sequenced for phylogenetic analysis, including 7 GI and 36 GII isolates. NoV GI isolates covered at least 5 genotypes, but no circulating strain predominated (online Appendix Figure 2, http://www.cdc.gov/ncidod/EID/vol12no08/06-0081-appG2.htm). For NoV GII isolates, we found ≥8 genotypes; GII/4 was the most prevalent (online Appendix Figure 3, http://www.cdc.gov/ncidod/EID/vol12no08/06-0081-appG3.htm).

To rule out the possibility of a quantitation artifact due to different stability between genogroups upon storage or freeze-thaw cycle, viral RNA was re-extracted and requantitated from a fecal specimen that had been stored for >6 months and coinfected with both NoV GI and GII. Repeat testing showed no drop in cDNA viral load for either genogroup, which suggests a comparable stability upon storage. Sequence complementarity among target, primers, and probe used in quantitation was also verified. While no sequence mismatch in primers and probe was found among the 7 NoV GI isolates, 5 of the 36 GII isolates had a single mismatch. Thus, the low cDNA viral load of NoV GI measured was unlikely due to sequence mismatching.

Conclusions

In this study, we show for the first time that the median cDNA viral load of NoV GII is ≥100-fold higher than that of GI in the fecal specimens of patients with NoV-associated gastroenteritis. Neither the possibility of quantitation artifacts as a result of primers and probe mismatching nor stability differences between genogroups on storage was likely to account for our observation. Moreover, 7 of 9 specimens with NoV GI/GII coinfection exhibited higher cDNA viral load of GII than that of GI. Also, the cDNA

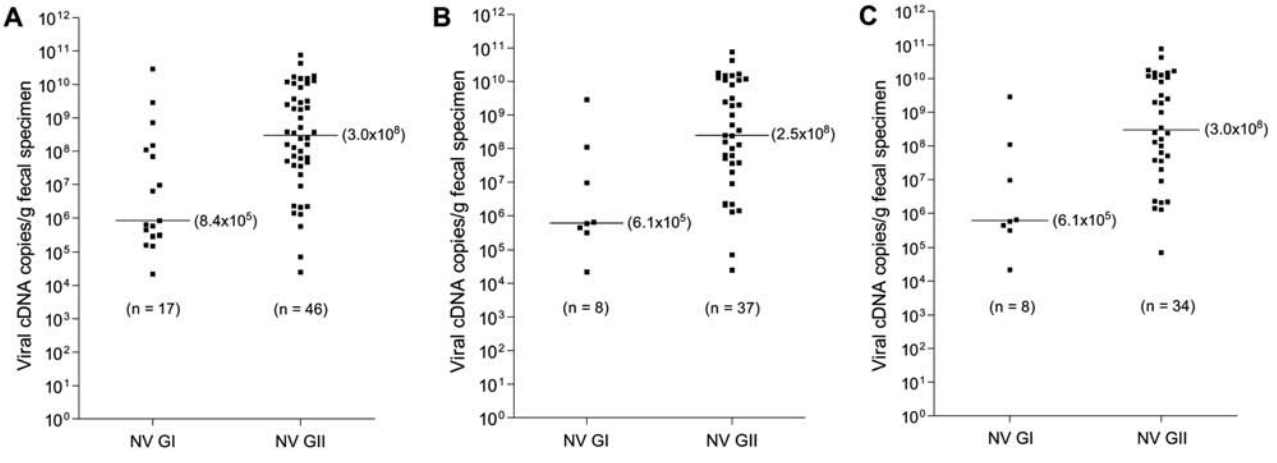

Figure 1. Scatterplots for cDNA viral load of noroviruses (NoV) genogroup I (GI) and GII. A) All positive isolates. B) All positive isolates, excluding those with NoV GI/GII coinfection. C) All positive isolates, excluding all those with viral coinfection (NoV GI and GII together with sapovirus, group A rotavirus, or both). The bars represent median cDNA viral loads. The p values were calculated by 2-tailed Mann-Whitney U test.

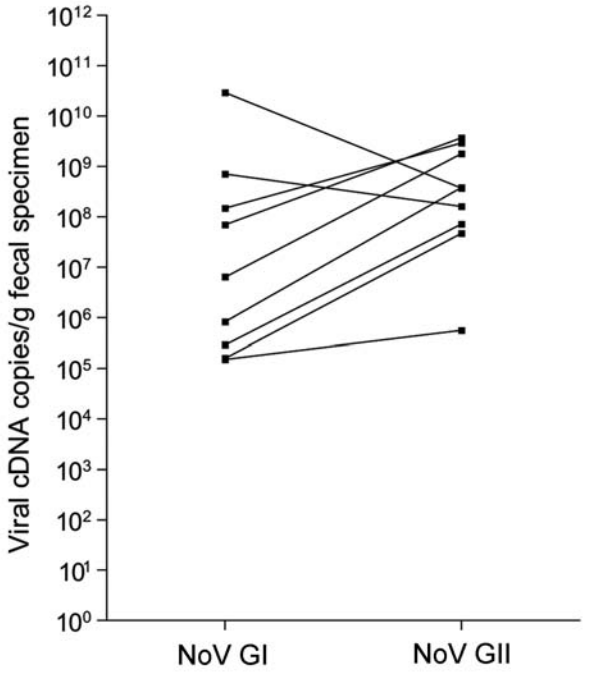

Figure 2. cDNA viral load in 9 specimens with norovirus (NoV) genogroup I (GI) and GII coinfection.

viral load of NoV GII was usually higher than that of GI for each collection month, a finding that further supports our interpretation.

We speculate that the increased cDNA viral load facilitates the transmission of NoV GII from infected persons to susceptible hosts through the fecal-oral route. Studies on other viruses have shown that viral load correlates well with the epidemiology of diseases. For example, the predominance of HIV-1 over HIV-2 has been suggested to be attributed to the higher viral load of HIV-1 (*8*). However, the implication of viral shedding pattern and cDNA viral load on epidemiologic characteristics and clinical manifestations of NoVs deserves further investigation. Our findings provide strong molecular evidence for the worldwide predominance of NoV GII and may open new research directions in the epidemiologic study of NoVs.

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References

1. Radford AD, Gaskell RM, Hart CA. Human norovirus infection and the lessons from animal caliciviruses. Curr Opin Infect Dis. 2004;17:471–8.
2. Zheng DP, Ando T, Fankhauser RL, Beard RS, Glass RI, Monroe SS. Norovirus classification and proposed strain nomenclature. Virology. 2006;346:312–23.
3. Koopmans M, Strien EV, Vennema H. Molecular epidemiology of human caliciviruses. In: Desselberger U, Gray J, editors. Viral gastroenteritis. London: Elsevier; 2003. p. 523–54.
4. Chan MC, Sung JJ, Lam RK, Chan PK, Lai RW, Leung WK. Sapovirus detection by quantitative real-time RT-PCR in clinical stool specimens. J Virol Methods. 2006;134:146–53.
5. Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, et al. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J Clin Microbiol. 2003;41:1548–57.
6. Pang XL, Lee B, Boroumand N, Leblanc B, Preiksaitis JK, Yu Ip CC. Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. J Med Virol. 2004;72:496–501.
7. Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, et al. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. J Clin Microbiol. 2004;42:2988–95.
8. De Cock KM, Adjorlolo G, Ekpini E, Sibailly T, Kouadio J, Maran M, et al. Epidemiology and transmission of HIV-2. Why there is no HIV-2 pandemic. JAMA. 1993;270:2083–6.

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Rickettsia felis in *Xenopsylla cheopis*, Java, Indonesia

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Rickettsia typhi and *R. felis*, etiologic agents of murine typhus and fleaborne spotted fever, respectively, were detected in Oriental rat fleas (*Xenopsylla cheopis*) collected from rodents and shrews in Java, Indonesia. We describe the first evidence of *R. felis* in Indonesia and naturally occurring *R. felis* in Oriental rat fleas.

Murine typhus (endemic typhus, fleaborne typhus), caused by *Rickettsia typhi*, is transmitted to humans by infected fleas and is relatively common wherever susceptible rodent hosts reside (*1*). Fleaborne spotted fever (cat flea typhus), caused by *Rickettsia felis*, is another zoonotic disease carried by fleas and appears to have an equally wide, cosmopolitan distribution; human infections with *R. felis* have a clinical syndrome similar to that of murine typhus (*1–4*). The cat flea, *Ctenocephalides felis*, has been identified as the primary arthropod vector of *R. felis* in North and South America (United States, Mexico, Peru, Brazil), Europe (Spain, France, United Kingdom, Cyprus), Africa (Gabon, Ethiopia), Asia (Thailand, Afghanistan, Israel), Australia, and New Zealand (*1,5–10*). We describe the first evidence of *R. felis* in Indonesia and apparent natural infections of *R. felis* in the Oriental rat flea, *Xenopsylla cheopis*, implicating this flea species for the first time as a potential vector for fleaborne spotted fever.

The Study

Samples of *X. cheopis* were collected from 39 live-captured, peridomestic rodents and shrews from 3 localities in Malang, East Java, Indonesia, during an epidemiologic study conducted in 1994 (*11*). In this study the fleas were reidentified by using morphologic criteria, stored in fresh 70% ethanol, and subsequently evaluated for the presence of rickettsial DNA. DNA sample preparations were derived from triturates of 103 individual fleas in 100 μL PrepManUltra sample preparation reagent (Applied Biosystems, Foster City, CA, USA). DNA preparations of 1 to 5 fleas collected from the same rodent were pooled for testing. Reaction mixtures for the quantitative real-time PCR (qPCR) assays had a total volume of 25 μL and contained 3 μL DNA template. The master mixes were prepared for the 17-kDa *Rickettsia*-, *R. typhi*– and *R. felis*–specific qPCR assays in a separate, clean (DNA-free) room as previously described (*6,12*). The primer and probe sequences for the 17-kDa *Rickettsia*-specific and *R. felis*–specific assays have been reported (*6,11*). The *R. typhi* forward (Rt557F: 5′-TGG TAT TAC TGC TCA ACA AGC T-3′) and reverse (Rt678R: 5′-CAG TAA AGT CTA TTG ATC CTA CAC C-3′) primers and probe (Rt640BP: 5′-TET-CGC GAT CGT TAA TAG CAG CAC CAG CAT TAT CGC G-DABCYL-3′) sequences are listed here. Included in each run were 3 negative controls (GIBCO Ultrapure DNA-free distilled water, Invitrogen Corporation, Grand Island, NY, USA), 1 produced in the clean room and 2 in a biosafety cabinet in another laboratory where DNA templates were added. A TOPO TA plasmid (Invitrogen Corporation) that contained the target sequence at 10^3 copies for each assay was used as a positive control. qPCR reactions were incubated in the SmartCycler (Cepheid, Sunnyvale, CA, USA) at 94°C for 2 min, followed by 50 cycles of a 2-step amplification protocol of 94°C for 5 s and 60°C for 30 s. Fluorescence was monitored during the annealing step of each cycle, and data were analyzed with SmartCycler software version 2.0c (Cepheid).

Rickettsial DNA was detected by 17-kDa qPCR in 7 of 39 pools containing 1–5 *X. cheopis* fleas. To determine whether *R. typhi* or *R. felis* infected these fleas, PCR assays specific for *R. typhi* and *R. felis ompB* partial sequence targets were performed (Table). Results of these assays showed that 5 of the 7 *Rickettsia*-positive *X. cheopis* fleas were infected with *R. typhi*, and 2 were positive for *R. felis*. The remainder of the 32 pools and all nontemplate controls were negative for *R. typhi* and *R. felis*. Additionally, 15 *Rickettsia*-free *C. felis* fleas (Heska Corporation, Loveland, CO, USA), evaluated at the same time and under the same conditions as the Malang fleas, were negative for *R. typhi* and *R. felis*.

Conclusions

To determine the identity of rickettsial agents infecting *X. cheopis* fleas collected from rodents and shrews in Malang, we assessed pools of 1 to 5 fleas from each animal. Our results confirm *R. typhi* in a known flea vector of murine typhus in a highly disease-endemic region of East Java, Indonesia (*11,13*). *R. felis* has been shown to infect fleas of peridomestic rodents (*7,8*) and fleas other than *C. felis* (*1,5,14*). However our report is the first of *R. felis* naturally infecting *X. cheopis* fleas, a vector of plague and

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Table. Detection of *Rickettsia typhi* and *Rickettsia felis* in flea pools by quantitative, real-time PCR, East Java, Indonesia

Location and distribution of rodents and shrews	No. flea pools (total no. fleas)*	No. positive pools (%)		
		17 kDa	*R. typhi*	*R. felis*
Rural (Mulyorejo)				
Rattus argentiventer (7.1%)	1 (2)	0	0	0
Rattus rattus (14.3%)	2 (3)	0	0	0
Rattus tiomanicus (57.1%)	1 (1)	0	0	0
Mus musculus (10.7%)	1 (2)	0	0	0
Chiropodomys gliroides (3.6%)	0			
Suncus murinus (7.1%)	0			
Total rural	5 (8)	0	0	0
Suburban (Bandungrejosari)				
Rattus exulans (3.2%)	1 (3)	0	0	0
R. rattus (74.2%)	13 (32)	1	0	1
R. tiomanicus (19.4%)	4 (9)	0	0	0
C. gliroides (3.2%)	1 (4)	0	0	0
Total suburban	19 (48)	1 (5.3)	0	1 (5.3)
Urban (Klojen)				
R. exulans (5.3%)	1 (4)	1	1	0
R. rattus (68.4%)	9 (28)	4	3	1
R. tiomanicus (10.5%)	2 (7)	0	0	0
Rattus sabanus (10.5%)	2 (5)	0	0	0
M. musculus (5.3%)	1 (3)	1	1	0
Total urban	15 (47)	6 (40)	5 (33.3)	1 (6.7)
Total	39 (103)	7 (18)	5 (12.8)	2 (5.1)

*Each pool of 1 to 5 fleas (*Xenopsylla cheopis*) came from a single animal.

murine typhus. Both *R. felis*–containing flea pools were derived from *Rattus rattus*, 1 from the suburban and 1 from the urban neighborhoods of Malang. *R. rattus* was the predominant species captured in urban and suburban environments (72%) and appears to be the primary host for *R. felis*– and *R. typhi*– infected *X. cheopis*. In the rural setting, where *R. rattus* was represented with far less frequency (14.3%), neither rickettsial agent was detected in collected fleas. These findings merit further epidemiologic investigation to better understand the relationship between *R. felis*, *R. typhi*, and *X. cheopis* and the transmission dynamics between flea and rodent.

Additionally, this report provides the first evidence of *R. felis* in the Indonesian archipelago. Investigations of rickettsial agents in Indonesia have been relatively few; to date, human infections with *R. felis* have not been reported from Indonesia. The lack of reports may be because a murine typhus–like disease associated with *R. felis* infection would not allow healthcare providers to clinically discriminate fleaborne spotted fever from murine typhus or other rickettsioses. In Indonesia, rickettsioses and typhoid fever are collectively referred to as *tifus*. Rickettsial tifus can be discerned by serologic tests or by observing when rickettsial tifus cases rapidly respond to treatment with a tetracycline or chloramphenicol. Furthermore, the inability to diagnose fleaborne spotted fever by laboratory means has been attributed to the cross-reactivity of antibodies to *R. felis* antigens with other rickettsial antigens (*1*). Consequently, serologic assays have been unable to differentiate fleaborne spotted fever from other rickettsioses. Thus, the high prevalence of murine typhus reported in Indonesia likely also includes fleaborne spotted fever. In addition, previously demonstrated serologic evidence of spotted fever group rickettsiae infection among residents of Gag Island, in eastern Indonesia (*15*), could have been due to *R. felis*. On the basis of data presented here and of recent reports of *R. felis* in other countries in Asia (*2–5,8,9*), healthcare providers in Indonesia should be alerted to the possibility of fleaborne spotted fever among their patients.

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References

1. Azad AF, Radulovic S. Higgins, Noden BH, Troyer JM. Flea-borne rickettsioses: ecological considerations. Emerg Infect Dis. 1997;3:319–27.
2. Parola P, Miller RS, McDaniel P, Telford SRIII, Rolain J-M, Wongsrichanalai C, et al. Emerging rickettsioses of the Thai-Myanmar border. Emerg Infect Dis. 2003;9:592–5.

3. Choi Y-J, Jang W-J, Kim J-H, Ryu J-S, Lee S-H, Park K-H, et al. Spotted fever group and typhus group rickettsioses in humans, South Korea. Emerg Infect Dis. 2005;11:237–44.
4. Phongmany S, Rolain J-M, Phetsouvanh R, Blacksell SD, Soukkhaseum V, Rasachack B, et al. Rickettsial infections and fever, Vientiane, Laos. Emerg Infect Dis. 2006;12:256–62.
5. Parola P, Sanogo OY, Lerdthusnee K, Zeaiter Z, Chauvancy G, Gonzalez JP, et al. Identification of *Rickettsia* spp. and *Bartonella* spp. in fleas from the Thai-Myanmar border. Ann N Y Acad Sci. 2003;990:173–81.
6. Blair PJ, Jiang J, Schoeler GB, Moron C, Anaya E, Céspedes M, et al. Characterization of spotted fever group rickettsiae in flea and tick specimens from northern Peru. J Clin Microbiol. 2004;42:4961–7.
7. Psaroulaki A, Antoniou M, Papaeustathiou A, Toumazos P, Loukaides F, Tselentis Y. First detection of *Rickettsia felis* in *Ctenocephalides felis* fleas parasitizing rats in Cyprus. Am J Trop Med Hyg. 2006;74:120–2.
8. Marié J-L, Fournier P-E, Rolain J-M, Briolant S, Davoust B, Raoult D. Molecular detection of *Bartonella quintana*, *B. elizabethae*, *B. koehlerae*, *B. doshiae*, *B. taylorii*, and *Rickettsia felis* in rodent fleas collected in Kabul, Afghanistan. Am J Trop Med Hyg. 2006;74:436–9.
9. Bauer O, Baneth G, Eshkol T, Shaw SE, Harrus S. Polygenic detection of *Rickettsia felis* in cat fleas (*Ctenocephalides felis*) from Israel. Am J Trop Med Hyg. 2006;74:444–8.
10. Schloderer D, Owen H, Clark P, Stenos J. Fenwick. *Rickettsia felis* in fleas, Western Australia. Emerg Infect Dis. 2006;12:841–3.
11. Richards AL, Soeatmandji DW, Widodo MA, Sardjono TW, Yanuwiadi B, Hernowati TE, et al. Seroepidemiological evidence for murine and scrub typhus in Malang, Indonesia. Am J Trop Med Hyg. 1997;57:91–5.
12. Jiang J, Chan TC, Temenak JJ, Dasch GA, Ching WM, Richards AL. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. Am J Trop Med Hyg. 2004;70:351–6.
13. Corwin AL, Soepranto W, Widodo PS, Rahardjo E, Kelly DJ, Dasch GA, et al. Surveillance of rickettsial infections in Indonesian military personnel during peace keeping operations in Cambodia. Am J Trop Med Hyg. 1997;57:569–70.
14. Stevenson HL, Labruna MB, Montenieri JA, Kosoy MY, Gage KL, Walker DH. Detection of *Rickettsia felis* in a New World flea species, *Anomiopsyllus nudata* (Siphonaptera: Ctenophthalmidae). J Med Entomol. 2005;42:163–7.
15. Richards AL, Ratiwayanto S, Rahardjo E, Kelly DJ, Dasch GA, Fryauff DJ, et al. Evidence of infection with ehrlichiae and spotted fever group rickettsiae among residents of Gag Island, Indonesia. Am J Trop Med Hyg. 2003;68:480–4.

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Avian Influenza among Waterfowl Hunters and Wildlife Professionals

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We report serologic evidence of avian influenza infection in 1 duck hunter and 2 wildlife professionals with extensive histories of wild waterfowl and game bird exposure. Two laboratory methods showed evidence of past infection with influenza A/H11N9, a less common virus strain in wild ducks, in these 3 persons.

Wild ducks, geese, and shorebirds are the natural reservoir for influenza A virus (*1*); all 16 hemagglutinin (H) and 9 neuraminidase (N) subtypes are found in these wild birds (*1*,*2*). Recently, the rapid spread of influenza A/H5N1 virus to new geographic regions, possibly by migrating waterfowl, has caused concern among public health officials who fear an influenza pandemic. Until now, serologic studies of the transmission of subtype H5N1 and other highly pathogenic strains of avian influenza have focused on humans who have contact with infected domestic poultry (*3*,*4*). In this cross-sectional seroprevalence study, we provide evidence of past influenza A/H11 infection in persons who were routinely, heavily exposed to wild ducks and geese through recreational activities (duck hunting) or through their employment (bird banding). To our knowledge, this study is the first to show direct transmission of influenza A viruses from wild birds to humans.

The Study

In mid-October 2004, we enrolled 39 duck hunters who were hunting in southeastern Iowa at Lake Odessa Wildlife Management Area, the state's only limited-access public waterfowl hunting area managed by the Iowa Department of Natural Resources (DNR). In February 2005 we enrolled 68 Iowa DNR employees, many of whom had duck hunted or had been involved annually in capturing and banding wild ducks and geese as part of their duties of employment. Ten (15%) of the 68 DNR workers reported no contact with ducks. The duck-hunting group consisted of men >16 years of age, and the DNR group consisted of 65 men and 3 women enrollees. The average age of the duck hunters and DNR workers was 34 and 47 years, respectively. The average number of years of waterfowl or bird exposure of the duck hunters and DNR workers was 19.8 and 21.5, respectively. In the 3 years before the study, influenza vaccine had been administered to 37% of the duck hunters and 35% of the DNR workers.

Microneutralization assay, adapted per Rowe et al. (*5*), was performed on all serum samples with influenza A subtypes H1 through H12 from avian sources. Virus at 100 $TCID_{50}$ (50% tissue culture infective dose)/50 μL was incubated at 37°C for 2 h with heat-inactivated serum in 96-well plates. One hundred microliters of trypsinized London MDCK cells at 2×10^5 cells/mL, grown to 70%–95% confluency, was added to each well. After 24 h at 37°C, the cells were acetone-fixed, and horseradish peroxidase–based ELISA was performed with mouse-specific anti-influenza A antibody. Optical density was read at 450 nm. All tested virus isolates were titrated with and without trypsin in the University of Iowa's Emerging Pathogens Laboratory; no significant difference in titers was observed. Backtiter controls were performed with each microneutralization assay.

Hemagglutination inhibition (HI) assay with horse erythrocytes, adapted per Meijer et al. (*6*), was performed on all hunter serum samples by using avian influenza A subtype H11. Heat-inactivated serum treated with receptor-destroying enzyme was first heme-adsorbed with packed horse erythrocytes. Serum was then incubated with virus at 8 hemagglutinin U/50 μL with 1% horse erythrocytes in 0.5% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature in V-bottom plates. The plates were then examined.

One 39-year-old duck hunter had a titer of 40, and 2 male DNR workers, ages 52 and 53, had titers of 10 against influenza A/H11N9/duck/Memphis/546/76 by microneutralization assay (Table). These 3 study participants had substantial lifetime exposures to wild waterfowl. The duck hunter and the 2 DNR workers had 31, 27, and 30 years of duck-hunting experience, respectively. The duck hunter spent 25–60 days in the marsh each year hunting ducks. He harvested 100 ducks annually and handled another 300 ducks with his hunting partners during the duck-hunting season from mid-September to early December. One of the positive DNR workers (age 52) had several years of live wild duck–banding exposure as part of his annual duties of employment, in addition to 27 years of duck-hunting exposure. Each year this wildlife professional had contact with >100 live ducks during the banding season in late August and early September. Serum

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Table. Serologic results and demographics of duck hunter and Iowa DNR workers*

Waterfowl handlers	Sex	Age, y	Hunting/bird exposure, y	MN titer	HI titer
Hunter	M	39	31	40	10
DNR 1	M	52	27	10	10†
DNR 2	M	53	30	10	10

*DNR, Department of Natural Resources; MN, microneutralization assay; HI, hemagglutination inhibition.
†Repeat HI titer was 20.

samples from all other study participants were negative against subtype H11N9 according to results of microneutralization assay and horse erythrocyte HI assays. The duck hunter's serum was not reactive to any other avian influenza hemagglutinin subtypes tested (H1–H10 and H12). The sera of the 2 H11-positive DNR workers had titers of 10 for influenza A/H2N2/mallard/NY/6750/78 according to microneutralization assay results and were negative for H1, H3–H10, and H12. Results of the H11 microneutralization assay were verified by horse erythrocyte HI assay that used subtype H11N9 virus. The titers by horse HI assay of the microneutralization assay–positive duck hunter and the 2 DNR workers were 10 or 20 (Table). These 3 study participants had not been vaccinated against influenza within 3 years before the study.

Conclusions

Virus transmission from wild waterfowl to humans has not been documented. To our knowledge this study is the first to assess hunters with substantial exposures to wild ducks and geese, the known natural reservoir of influenza A virus in nature (*1*). During late August and early September in Iowa, when the banding of wild ducks occurs, and in mid-September, when duck hunting begins, a significant proportion of hatch-year mallards (up to 65%) and other ducks may be infected with influenza A virus according to other studies in North America (*1,7*). Later in the season, as the duck migration progresses, a decrease in prevalence is commonly seen (*1,8*). In late August 2004, we isolated influenza virus from mallards (60%) and from wood ducks (13%) in Iowa (data not shown).

Even though the H11-positive study participants had several years of exposure to wild birds infected with avian influenza virus through hunting and duck banding, they did not wear personal protective equipment, such as gloves, masks, or eye protection. These participants also did not use tobacco, a recently identified risk factor among swine facility workers with elevated serum antibodies against swine strains of influenza (*9*).

In this study we did not attempt to associate disease symptoms with exposure to wild waterfowl. Others have shown that domestic bird–acquired influenza A/H7N7 in humans may frequently lead to minor illness, such as conjunctivitis (*4,10,11*), although more serious disease has been recorded (*4,10*). We provide serologic evidence from 2 assays, microneutralization assay and horse erythrocyte HI, for past infection in humans with avian influenza A/H11 and no other avian influenza subtypes. Our findings are consistent with those of Beare and Webster (*12*), who reported a lack of antibody response in human volunteers inoculated with avian influenza strains with HA antigens wholly alien to humans. Those researchers did not inoculate volunteers with H11. In our study, a less common hemagglutinin subtype (H11) has apparently caused serologically detectable infections in high-exposure groups, whereas the more common hemagglutinin subtypes H4 and H6 (*13–15*) in wild ducks have not. The reason for this finding is unknown but may include the following: 1) H11 may have increased ability to infect humans, 2) H11 may provoke a relatively strong and detectable immune response, and 3) our serologic assays may be more sensitive in detecting H11 infection than other H subtypes.

Even though none of the H11-positive study participants had received influenza vaccine within the previous 3 years, the 2 positive DNR workers also showed reactivity by microneutralization assay to avian subtype H2N2. This result was not unexpected and likely represents reactivity from natural infection of the human H2N2 strain derived from avian sources that circulated from 1957 to 1967. Forty-one percent of participants of similar age (range 43–68 years, average 56 years) who grew up during the era of the human H2N2 pandemic also had positive test results. Except for the 2 H11N9-positive DNR workers, the other H2N2-positive study participants were nonreactive against avian subtype H11N9 (data not shown). This finding strengthens our conclusion that there was no cross-reactivity between H2N2 and H11N9 antisera. None of H11-positive study participants was reactive to avian subtypes H1 or H3, although others in the study population were. Only 7% and 18% of the study population were reactive by microneutralization assay against H1 and H3, respectively.

The relative lack of antibody response in our study population, who had substantial exposures to waterfowl with influenza A infections, and in inoculated volunteers from Beare and Webster (*12*) suggests that avian influenza infections in humans exposed to wild waterfowl may occur more commonly than we are able to detect with current methods. Although the sample size of our study was relatively small, our results suggest that handling wild waterfowl, especially ducks, is a risk factor for direct transmission of avian influenza virus to humans.

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Dr Gill, in addition to providing emergency room duties as a physician, maintains an active research program as the zoonotic disease specialist at the University of Iowa Hygienic Laboratory. He recently codiscovered a new species of spotted fever group rickettsia and relapsing fever borrelia in the bat tick, *Carios kelleyi*. He also holds an adjunct position in the Department of Epidemiology in the College of Public Health at the University of Iowa.

References

1. Stallknecht DE, Shane SM. Host range of avian influenza virus in free-living birds. Vet Res Commun. 1988;12:125–41.
2. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol. 2005;79:2814–22.
3. Bridges CB, Lim W, Hu-Primmer J, Sims L, Fukuda K, Mak KH, et al. Risk of influenza A (H5N1) infection among poultry workers, Hong Kong, 1997–1998. J Infect Dis. 2002;185:1005–10.
4. Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, et al. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet. 2004;363:587–93.
5. Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W, et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J Clin Microbiol. 1999;37:937–43.
6. Meijer A, Bosman A, van de Kamp EE, Wilbrink B, van Beest Holle Mdu R, Koopmans M. Measurement of antibodies to avian influenza virus A (H7N7) in humans by hemagglutination inhibition test. J Virol Methods. 2006;132:113–20.
7. Hinshaw VS, Wood JM, Webster RG, Deibel R, Turner B. Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas in North America. Bull World Health Organ. 1985;63:711–9.
8. Stallknecht DE, Shane SM, Zwank PJ, Senne DA, Kearney MT. Avian influenza viruses from migratory and resident ducks of coastal Louisiana. Avian Dis. 1990;34:398–405.
9. Ramirez A, Capuano AW, Wellman DA, Lesher KA, Setterquist SF, Gray GC. Preventing zoonotic influenza infection. Emerg Infect Dis. 2006;12:997–1000.
10. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekmen JM, Kemink SA, Munster V, et al. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A. 2004;101:1356–61.
11. Kurtz J, Manvell RJ, Banks J. Avian influenza virus isolated from a woman with conjunctivitis. Lancet. 1996;348:901–2.
12. Beare AS, Webster RG. Replication of avian influenza viruses in humans. Arch Virol. 1991:119:37–42.
13. Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, et al. Influenza A viruses of migrating wild aquatic birds in North America. Vector-Borne Zoonotic Dis. 2004;4:177–89.
14. Hanson BA, Stallknecht DE, Swayne DE, Lewis LA, Senne DA. Avian influenza viruses in Minnesota ducks during 1998–2000. Avian Dis. 2003; 47:867–71.
15. Slemons RD, Hansen WR, Converse KA, Senne DA. Type A influenza virus surveillance in free-flying, nonmigratory ducks residing on the eastern shore of Maryland. Avian Dis. 2003;47:1107–10.

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OFFLU Network on Avian Influenza

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OFFLU is the name of the network of avian influenza expertise inaugurated jointly in 2005 by the Food and Agriculture Organization of the United Nations and the World Organisation for Animal Health. Achievements and constraints to date and plans for the future are described.

Many bird species are susceptible to infection with influenza A viruses. Aquatic birds form a major reservoir; they harbor representatives of all 16 hemagglutinin subtypes and 9 neuraminidase subtypes (*1*). Most of these viruses are of low pathogenicity, whether in wild or domesticated bird species, and most do not cross-infect mammals. Strains of virus that are highly pathogenic for domestic poultry (chickens and turkeys) emerge from time to time and can cause epidemics with high death rates in affected flocks; clinical signs and pathologic lesions have been described (*2*). These highly pathogenic strains are almost invariably of hemagglutinin types 5 or 7 (H5 or H7), although low-pathogenic strains of H5 and H7 also circulate in wild birds and can infect poultry.

Outbreaks of H5 or H7 highly pathogenic avian influenza have occurred in recent years in different countries, including Italy, the Netherlands, Canada, and Mexico, but the most notable global event has been the progressive spread of highly pathogenic H5N1 virus, originally in Asia, then extending into parts of Europe and Africa. A feature of this virus is its ability to infect and kill human hosts, although so far it appears not to have spread substantially from human to human, apart from some possible family clusters. This spread has led to the mobilization of World Health Organization (WHO) and medical virology forces worldwide to monitor the perceived threat of a potential human pandemic (*3*).

For all its importance as a zoonotic threat, highly pathogenic avian influenza is a devastating disease of domestic poultry, which is one of the world's primary sources of animal protein, and the economic effects on livestock producers and rural communities in affected countries are enormous. All strains of H5 and H7 avian influenza viruses, plus any other H subtypes that show pathogenic traits in poultry, are formally notifiable by national veterinary authorities to the World Organisation for Animal Health (OIE) (*4*). Highly pathogenic avian influenza is among the transboundary animal diseases, which are a major concern for international organizations involved in disease surveillance and control, namely OIE and the Food and Agriculture Organization of the United Nations (FAO) (*5*).

Recognizing the global threat posed, particularly by the H5N1 epidemic, the international organizations OIE and FAO agreed in 2005 to establish a network of expertise to support international efforts to monitor and control this disease in poultry and other bird species. The network was designed from the start to interface with the existing WHO influenza network, which was focused on the threat to human health. The new animal influenza network was named OFFLU.

The originally stated objectives of OFFLU were to develop research on avian influenza, offer advice and veterinary expertise to member countries, and collaborate with the WHO animal influenza network. In the year since the network was established, the highest priority tasks have become exchanging scientific data and virus isolates (both within OFFLU and in liaison with the WHO network) and providing experts to assist with missions to affected countries. Developing research activities remains an essential need and will be pursued by individual participant institutions, either alone or in partnerships, but it is not the highest priority task for OFFLU itself.

The core of OFFLU is its scientific committee. Its members represent most of the world's expertise on avian influenza. The activities of the committee and the network are directed by a steering committee that represents key interests of the partner organizations, FAO and OIE. The network is supported by a secretariat, currently located at Padova, Italy, at an OIE/FAO reference center on avian influenza. The network has a philosophy of openness and is keen to involve scientific collaborators from as wide a field as possible. The core network is built around the OIE and FAO reference laboratories for avian influenza, but it is not limited to laboratories. Epidemiologists in particular have a role to play. Qualified persons and institutions are invited to register with the network as scientific collaborators. In addition, the network seeks to establish links with field experts with knowledge and experience of the global poultry industry and the control of infectious diseases, as well as with ornithologists and experts in wildlife diseases.

The relevance of OFFLU was recognized at the Beijing avian influenza pledging conference (*6*). However, funding is needed to empower and sustain this network. Without specific funding, its effectiveness will be limited. Veterinary and scientific expertise on avian influenza is in short supply given the scale of the current epidemic. Requests to FAO and OIE for expert missions to affected countries arrive on a regular basis, and demand exceeds supply. By establishing and extending the network, we hope that these pressures can be mitigated and needs, particularly for developing countries, can be adequately met.

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OFFLU itself does not send missions, but it provides a cadre of expertise to organizations that undertake this task.

The current priorities of OFFLU are to promote the collection, exchange, and characterization of animal influenza viruses, including the deposition of sequence data in genome banks. A good start has been made on this process, and it is a priority for further development. In addition, the network will seek to establish linkages between laboratories in industrialized and developing countries to provide capacity building and training. The efforts to supply consistent, coordinated advice, expertise, and technical assistance to infected countries will continue. Research will continue on numerous fronts and will address scientific questions such as the molecular basis for virulence, factors involved in host specificity, basic and applied immunology including vaccine development, epidemiology, evaluation of control strategies, and the development of better diagnostic tests.

The network is facing several constraints, including heavy demand on limited resources, regulatory issues (e.g., problems with shipping viruses between countries), and the inevitable tensions between sharing information as a public good versus the desire to protect intellectual property rights. But after only 1 year in operation, the OFFLU network is already proving its effectiveness. It has an active website (www.offlu.net) as a primary means of communication, and it is well placed to channel the world's limited scientific resources to best effect in tackling this disease, which is of such great international concern for both animal and human health.

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

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References

1. World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals. 5th ed. 2005 Jul 8 [cited 2006 Jun 7]. Available from http://www.oie.int/eng/normes/mmanual/A_00037.htm
2. Capua I, Mutinelli F. A colour atlas and text on avian influenza. Bologna, Italy: Papi Editore; 2001.
3. World Health Organization. Avian influenza. [cited 2006 Jun 7]. Available from http://www.who.int/csr/disease/avian_influenza/en/index.html
4. World Organisation for Animal Health. Terrestrial animal health code. 14th ed. 2005 Jul 27 [cited 2006 Jun 7]. Available from http://www.oie.int/eng/normes/mcode/en_chapitre_2.7.12.htm
5. Food and Agriculture Organization, World Organisation for Animal Health, and the World Health Organization. A global strategy for the progressive control of highly pathogenic avian influenza (HPAI). 2005 Nov [cited 2006 Jun 7]. Available from http://www.fao.org/ag/againfo/subjects/documents/ai/HPAIGlobalStrategy31Oct05.pdf
6. Food and Agriculture Organization. Proposal for a global programme: avian influenza control and eradication. 2006 Mar 17 [cited 2006 Jun 7]. Available from http://www.fao.org/ag/againfo/subjects/documents/ai/Global_Programme_Jan06.pdf

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Salmonella Typhimurium DT104, Italy

To the Editor: The recent article by Helms et al. described the distribution of *Salmonella enterica* serovar Typhimurium definitive page type 104 (DT104) infections in 29 countries from 1992 to 2001 (*1*). Results from Italy were not presented because routine phage typing was not performed before 2001. Since 2002, circulation of *S.* Typhimurium phage types has been monitored by the laboratory-based surveillance system Enter-net Italia, which was coordinated by Istituto Superiore di Sanità as part of the European network for the surveillance of foodborne infections (*2*). From 2000 to 2004, *S.* Typhimurium accounted for ≈40% of all human *Salmonella* isolates each year. Since 2002, ≈20% of the *S.* Typhimurium isolates were identified as DT104, and all had a pentavalent resistance pattern (resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) (*3*). Although the results reported by Helms et al. (*1*) refer to a different period (1992–2001), the Italian data are similar to those from many other countries in northern and western Europe.

According to the Colindale scheme for phage typing ([*4*] and L.R. Ward, pers. comm.), numerous distinguishable DT104 subtypes can be identified as DT104 A, B, C, H, and L. Most (90%) *S.* Typhimurium DT104 strains isolated during the last 2 years belonged to subtype DT104L.

Emergence of phage subtype DT104A was identified in June 2004 during an outbreak of salmonellosis in Rome. This subtype had never been previously identified in Italy. All DT104A isolates were susceptible to the Enter-net panel of antimicrobial drugs (*2*), a feature unusual for *S.* Typhimurium (*5*). A total of 63 cases were confirmed; 61 were from Rome, and 2 were from a neighboring region. All isolates had similar pulsed-field gel electrophoretic profiles when analyzed with the Salm-gene protocol (*6*). Since the outbreak, 1 additional human isolate of DT104A was identified from a resident of the same neighboring region. This isolate was also susceptible to the panel of antimicrobial drugs. A fermented pork salami was epidemiologically implicated as the vehicle of infection. No microbiologic evidence was found because no food samples were available when the outbreak was recognized.

The incidence of DT104 in Italy has remained stable from 2002 through 2004. However, emergence of subtype DT104A during a recent outbreak highlights the need for subtyping in identifying communitywide outbreaks and in monitoring changing subtype patterns.

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References

1. Helms M, Ethelberg S, Mølbak K, DT104 Study Group. International *Salmonella* Typhimurium DT104 infections, 1992–2001. Emerg Infect Dis. 2005;11:859–67.
2. Enter-net. International surveillance network for the enteric infections *Salmonella* and VTEC O157. [cited 2006 May 12]. Available from http://www.hpa.org.uk/hpa/inter/enter-net_menu.htm
3. Busani L, Graziani C, Battisti A, Franco A, Ricci A, Vio D, et al. Antibotic resistance in *Salmonella enterica* serotypes Typhimurium, Enteritidis and Infantis from human infections foodstuffs and farm animals in Italy. Epidemiol Infect. 2004;132:245–51.
4. Anderson ES, Ward LR, De Saxe MJ, Old DC, Barker R, Duguid JP. Bacteriophage-typing designations of *Salmonella* Typhimurium. J Hyg (Lond). 1977;78: 297–300.
5. Malorny B, Schroeter A, Bunge C, Helmuth R. Prevalence of *Escherichia coli* O157:H7 prophage-like sequences among German *Salmonella enterica* serotype Typhimurium phage types and their use in detection of phage type DT104 by polymerase chain reaction. Vet Microbiol. 2002;87:253–65.
6. Peters TM, Maguire C, Threlfall EJ, Fisher IST, Gill N, Gatto AJ. on behalf of the Salm-gene project participants. The Salm-gene project—a European collaboration for DNA fingerprinting for food-related salmonellosis. Euro Surveill. 2003;8:46–50.

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Echovirus 13 Aseptic Meningitis, Brazil

To the Editor: Human enteroviruses (polioviruses, coxsackievirus A, coxsackievirus B, echoviruses, enterovirus 71, and newer recognized serotypes) belong to the *Picornaviridae* family, *Enterovirus* genus (*1*). They are common viral agents associated with a diversity of clinical manifestations, including respiratory illness; nonspecific rashes; hand, foot, and mouth disease; myocarditis; acute hemorrhagic conjunctivitis; and central nervous system (CNS) syndromes (*2*). Acute viral infections of the CNS are the source of a group of globally distributed diseases, which affect the population in a sporadic, endemic, or epidemic way. These infections cause a number of illnesses, particularly in children, and may result in serious sequelae; in severe cases, they can be fatal (*3*). Meningitis, encephalitis, acute flaccid paralysis (poliomyelitis), mononeuritis, polyneuritis, and Reye syndrome constitute most of the illnesses (*4*). Nonpolio enteroviruses are responsible for >80% of viral meningitis cases in which the etiologic agent

is identified (*2*). Several of the 28 currently recognized serotypes of echovirus are found in association with these infections (*3*).

We describe an outbreak of aseptic meningitis that occurred in southern Brazil in 2003 with echovirus 13 (E13) virus as the etiologic agent. This is the first meningitis outbreak due to E13 reported in the country.

From March to April 2003, 17 children and young adults from Horizontina City (population 16,800), Rio Grande do Sul State, southern Brazil, with symptoms of meningitis, sought medical attention at the local hospital. Seven of these case-patients were linked to each other either by school or domiciliary contact. Lumbar puncture showed clear cerebrospinal fluid (CSF), which suggests a viral cause. The following symptoms were associated with patients: fever (92%), headache (84%), vomiting (79%), diarrhea, stiff neck, and fatigue (7.69% each). Patients' ages ranged from 1 to 19 years of age, with the age peak incidence in children 5–9 years of age (46%). Fifty-eight percent of patients were male. All patients recovered, and no sequelae or deaths were identified. The pattern of meningitis associated with E13 in this outbreak was clinically similar to those observed in aseptic meningitis due to other enteroviruses in previous outbreaks.

For diagnostic purposes, 12 CSF and 8 fecal specimens were collected from the 17 patients with clinically suspected viral meningitis. For viral diagnosis, RD and HEp2 cells were injected with 0.2 mL of each clinical specimen (clarified fecal specimens and CSF) and examined daily for at least 7 days postinoculation. Enterovirus characteristic cytopathic effect was observed in 6 (50%) of 12 CSF samples and in 5 (62.5%) of 8 fecal samples. All isolates were typed as echovirus 13 by a reverse transcription–PCR and nucleotide sequencing of a portion of the VP1 gene (*5*).

Before 2000, echovirus 13 was considered a rare serotype of enterovirus (*6*) and had never been reported in association with outbreaks (*7*). In the United States, before 2001, this enterovirus accounted for only 65 of the 45,000 reported enteroviral isolates (*6*). However, the incidence of E13 is increasing; several meningitis outbreaks have been recently reported in England, Germany, Belgium, Spain, France, Israel, and Japan (*8*).

In spite of the temporal clustering and close contact of 7 patients, the causes of the outbreak were not completely defined and remain speculative. The sudden emergence of E13 as a prominent enterovirus associated with viral meningitis in many countries, including Brazil, demonstrates the potential of enteroviruses to circulate widely and to unpredictably cause diseases, which underscores the continued need for enterovirus surveillance.

Although this specific outbreak was restricted both geographically and in terms of magnitude (only 17 cases), E13 seemed to be widely distributed in Brazil and has been detected in fecal specimens obtained from patients with acute flaccid paralysis since 1998 (C. Blal, unpub. data). Epidemiologic surveillance plays a crucial role in understanding the changing patterns of enterovirus infection and disease associations. Such knowledge may help in the control of diseases (*9,10*). Although identifying the enterovirus serotype does not contribute substantially to patient management, establishing the dominant virus each year or in each outbreak is essential for epidemiologic purposes.

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References

1. King AMQ, Brown F, Christian P, Hovi T, Hyypia T, Knowles NJ, et al. Family *Picornaviridae*. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, et al., editors. Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses. San Diego: Academic Press; 2000. p. 657–83.
2. Morens DM, Pallansch MA. Epidemiology. In: Rotbart HA, editor. Human enterovirus infections. Washington: ASM Press; 1995. p. 3–23.
3. Melnick JL. Enterovirus: polioviruses, coxsackievirus, echoviruses and newer enteroviruses. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, et al., editors. Fields virology, 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1996. p. 655–712.
4. Alexander JP, Baden L, Pallansch MA, Anderson LJ. Enterovirus 71 infections and neurologic disease—United States, 1977–1991. J Infect Dis. 1994;169:905–8.
5. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of human enteroviruses by partial sequencing of VP1. J Clin Microbiol. 1999;37:1288–93.
6. Centers for Disease Control and Prevention. Echovirus type 13—United States, 2001. MMWR Morb Mortal Wkly Rep. 2001;50:777–80.
7. Moore M. Enteroviral disease in the United States, 1970–1979. J Infect Dis. 1982;146: 103–8.
8. Mullins JA, Khetsuriani N, Nix WA, Oberste MS, LaMonte A, Kilpatrick DR, et al. Emergence of echovirus type 13 as a prominent enterovirus. Clin Infect Dis. 2004;38:70–7.

9. McIntyre JP, Keen GA. Laboratory surveillance of viral meningitis by examination of cerebrospinal fluid in Cape Town, 1981–9. Epidemiol Infect. 1993;111:357–71.
10. Hovi T, Stenvik M, Rosenlew M. Relative abundance of enterovirus serotypes in sewage differs from that in patients: clinical and epidemiological implications. Epidemiol Infect. 1996;116:91–7.

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Nonsteroidal Antiinflammatory Drugs and Group A Streptococcal Infection

To the Editor: Factor et al. recently reported the results of a population-based, case-control study regarding risk factors for pediatric invasive group A streptococcal (GAS) infection (*1*), noting that the "new" use of nonsteroidal antiinflammatory drugs (NSAIDs), defined as NSAID use <2 weeks before diagnosis, was associated with invasive GAS infection, whereas self-defined "regular" NSAID use was not. The control population consisted of nonhospitalized, age-matched children contacted by telephone (*1*). Although we endorse the authors' conclusion that, "…the measurements of new use and regular use [of NSAIDs] are too crude to clearly identify their role as a risk factor," a more detailed discussion of their findings and conclusions is warranted.

Because of their antiinflammatory effects, NSAIDs have been suspected of suppressing host immunity during infection, particularly GAS infection (*2*). However, determining a causal association between NSAID use and infectious diseases has been problematic, especially when using retrospective studies (*3*). The results of such observational studies often suffer from protopathic bias, in which drugs are applied to treat symptoms that are actually early manifestations of the outcome of interest (*4*). Consequently, rather than being a direct determinant (i.e., causative risk factor) for invasive GAS infection, NSAID use could mark the onset of disease symptoms (fever, localized pain, and inflammation). Therefore, because of protopathic bias, the study by Factor et al. had a substantial chance of identifying an association between NSAID use and invasive GAS infection a priori.

Neither the fact that patients in the study by Factor et al. received NSAIDs any time during the 2 weeks before the diagnosis of invasive GAS infection nor the finding that nonhospitalized children (controls) were unlikely to have received NSAIDs in the 2 weeks before their interview should be surprising. A more informative case-control study would have matched case-patients with similar-aged children who had febrile infections not caused by GAS infection; both groups of children would have been equally likely to have received analgesic and antipyretic medications. Furthermore, population-based data suggest that most patients with invasive GAS infection are hospitalized (*5*), so hospital-based controls, rather than population controls, might have provided a more appropriate comparison group.

Prospective studies have failed to define a causal link between NSAIDs and invasive GAS infections (*3*), though such studies were not specifically designed to investigate this relationship. To best test the hypothesis that NSAIDs increase the risk for invasive GAS infection, a randomized, prospective trial should be done. Such a trial is unlikely to take place, however, because of questionable ethics and because the sample necessary to detect a significant difference would be prohibitively large.

Although NSAIDs may neither alter the risk of developing an invasive GAS infection nor accelerate an established infection, these drugs can mollify the signs and symptoms of streptococcal infection, possibly delaying appropriate management and treatment (*3*). However, the potential adverse consequences of suppressing clinical indicators of disease severity (e.g., fever, pain, and inflammation) with NSAIDs apply to myriad infectious and inflammatory conditions, not just invasive streptococcal disease.

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References

1. Factor SH, Levine OS, Harrison LH, Farley MM, McGeer A, Skoff T, et al. Risk factors for pediatric invasive group A streptococcal disease. Emerg Infect Dis. 2005;11:1062–6.
2. Stevens DL. Could nonsteroidal antiinflammatory drugs (NSAIDs) enhance the progression of bacterial infections to toxic shock syndrome? Clin Infect Dis. 1995;21:977–80.
3. Aronoff DM, Bloch KC. Assessing the relationship between the use of nonsteroidal antiinflammatory drugs and necrotizing fasciitis caused by group A streptococcus. Medicine (Baltimore). 2003;82:225–35.
4. Signorello LB, McLaughlin JK, Lipworth L, Friis S, Sorensen HT, Blot WJ. Confounding by indication in epidemiologic studies of commonly used analgesics. Am J Ther. 2002;9:199–205.
5. Mulla ZD, Leaverton PE, Wiersma ST. Invasive group A streptococcal infections in Florida. South Med J. 2003;96:968–73.

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Detecting *Clostridium botulinum*

To the Editor: In the October 2005 issue of Emerging Infectious Diseases, Song et al. described a fiber-optic, microsphere-based, high-density array composed of 18 species-specific probe microsensors, used to identify biological warfare agents, including *Clostridium botulinum* (*1*). Although the researchers used multiple probes for *C. botulinum*, we doubt that this approach is suitable for this organism.

C. botulinum comprises a heterogenous group of subspecies that produce botulinum neurotoxin (BoNT); identification and characterization usually rely on animal testing that focuses on antigenetically distinct toxins (*2*). Although strains of *C. botulinum* that do not produce toxins are sometimes isolated from wound infections not related to botulism, some strains of *C. butyricum* and *C. baratii* are also able to produce BoNTs.

The mouse bioassay is currently the accepted method for detecting BoNT. In this assay, mice that receive an intraperitoneal injection containing a sample with more than a minimum lethal dose show symptoms of botulinum intoxication and die. ELISAs, which recognize protein antigenic sites, are still less sensitive than the mouse bioassay (*3*).

Because the mouse bioassay requires euthanizing many animals, and results are not available for several hours, new diagnostic methods are needed. For *C. botulinum*, an organism widely dispersed in the environment, DNA-based methods may not provide the ultimate solution. Rapid methods to detect and differentiate active BoNTs, such as the rapid, mass spectrometry-based, functional method, are promising candidates to substitute for animal testing in the near future (*4*).

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References

1. Song L, Ahn S, Walt DR. Detecting biological warfare agents. Emerg Infect Dis. 2005;11:1629–32.
2. Grif K, Dierich MP, Much P, Hofer E, Allerberger F. Identifying and subtyping species of dangerous pathogens by automated ribotyping. Diagn Microbiol Infect Dis. 2003;47:313–20.
3. Ferreira JL, Eliasberg SJ, Edmonds P, Harrison MA. Comparison of the mouse bioassay and enzyme-linked immunosorbent assay procedures for the detection of type A botulinal toxin in food. J Food Prot. 2004;67:203–6.
4. Barr JR, Moura H, Boyer AE, Woolfitt AR, Kalb SR, Pavlopoulos A, et al. Botulinum neurotoxin detection and differentiation by mass spectrometry. Emerg Infect Dis. 2005;11:1578–83.

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Echinococcus multilocularis in Dogs, Japan

To the Editor: Alveolar echinococcosis in humans is endemic in Japan; however, the causal agent, *Echinococcus multilocularis*, has been restricted to the northernmost insular prefecture of Hokkaido, where the Tsugaru Strait acts as a natural physical barrier against migration to the mainland. Two *E. multilocularis* invasions into Hokkaido have occurred (*1*). The first invasion to the offshore island of Rebun in the mid-1920s was successfully controlled; however, the second invasion, supposedly in the 1940s, led to the current epidemic on the main island of Hokkaido. Both invasions were entirely or partly caused by humans who removed foxes from disease-endemic areas without taking the necessary precautions.

The finding of 19 autochthonously acquired cases of alveolar echinococcosis in prefectures other than Hokkaido (*2*) implies that the parasite exists in other areas, although the source of infection has yet to be identified. In many countries, studies of the increased spread of the parasite have traditionally focused on the contribution of foxes (*3*); however, these cases may also have been spread by domestic dogs from disease-endemic areas. Dogs are susceptible to infection with the parasite from rodents. Although the prevalence of *E. multilocularis* among dogs in Hokkaido is certainly lower than that in foxes (*4–6*), dogs can traverse considerably greater distances by various modes of transport. The number of dogs that travel from Hokkaido to other prefectures has been estimated at >12,000 per year (*7*). Although dogs may carry the parasite to remote areas, surveys of population dynamics have not been undertaken. We therefore studied the extent of *E. multilocularis* infection in dogs being transported by their owners from 4 ferry ports in Hokkaido (Hakodate, Muroran, Otaru, and Tomakomai) from September 2003 through October 2004.

We tested 183 fecal samples from 41 resident (in Hokkaido) and 142 nonresident dogs. We screened for the *Echinococcus*-specific coproantigen by using a commercial enzyme-linked immunosorbent assay kit (CHEKIT-Echinotest, Bommeli Diagnostics, Liebefeld-Bern, Switzerland) and following the manufacturer's recommendations. One dog from each group had the *Echinococcus* coproantigen. To confirm the specificity of the results, these 2 dogs were treated with 1 oral dose of praziquantel, 5 mg/kg.

Subsequent fecal samples were subjected to coproantigen testing and specific PCR amplification according to the method of Dinkel et al. (*8*). The coproantigen test showed a significant reduction in the optical density value for both dogs, which can be interpreted as effective deworming for *Echinococcus*. However, different results were obtained for the PCR test, in which assays of fecal samples from the nonresident dog during the second round of nested PCR produced a single band of the expected size (Figure). Direct sequencing showed that the band was the same as bands obtained for *E. multilocularis* isolates from Hokkaido (GenBank accession no. AB243207). Conversely, fecal samples from the resident dog did not yield any positive PCR results.

The reason for the discrepancy is unclear, but it may be a false reaction in either test. Given that a reduced optical density value was obtained after administration of the taeniacidal drug, the false-positive result of the coproantigen test might have been caused by another taeniid species. Such cross-reaction has been reported previously with this test (*9*). However, no worm debris was found in the fecal samples. Alternatively, sexual maturation or low infection intensity of *E. multilocularis* may produce false-negative results in PCR assays (*8*). Thus, because the owner stated that the dog was allowed to roam freely and frequently preyed on rodents, this coproantigen-positive but coproDNA-negative dog was highly suspected of being infected with *E. multilocularis*.

Infection among wild foxes can spread to domestic dogs by way of highly contaminated rodent hosts (*10*). A nonresident dog became infected with *E. multilocularis* despite staying in Hokkaido for only 5 days and being permitted to roam freely for just a few hours. This finding suggests a high infection pressure of *E. multilocularis* to domestic dogs within the area. In addition, the increased popularity of keeping dogs as companions, greater frequency of dogs' traveling with their owners, and high prevalence in foxes from urban and rural areas in Hokkaido (*5*,*6*) all contribute to the possibility that *E. multilocularis* could emerge in unsuspected locations. Thus, to prevent this parasite from spreading, measures such as those used by the Pet Travel Scheme of the United Kingdom should be applied to ensure that dogs from disease-endemic areas are pretreated before entry to the main island of Japan.

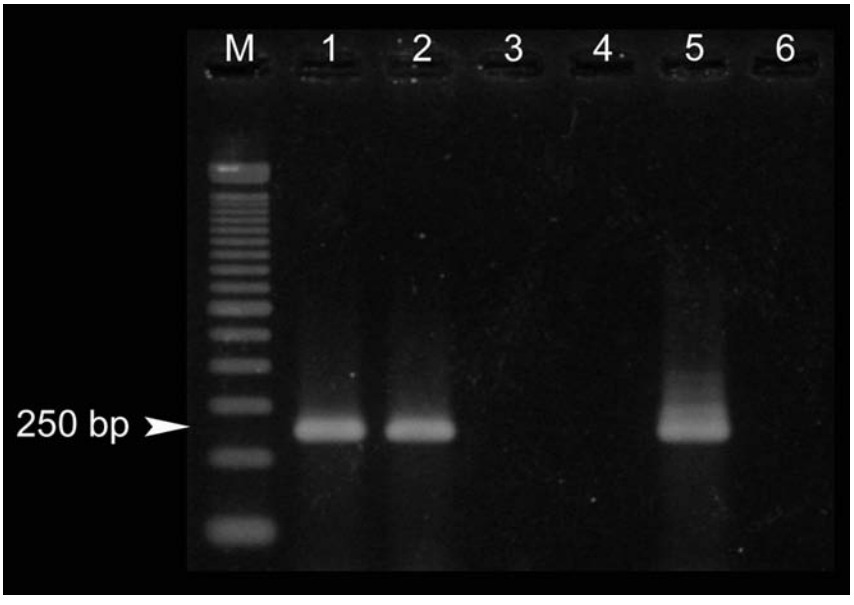

Figure. Nested PCR amplification of coproDNA from 2 coproantigen-positive dogs. Lane M, size marker (100-bp ladder); lane 1, nonresident dog (before treatment); lane 2, nonresident dog (1 day after treatment); lane 3, resident dog (before treatment); lane 4, resident dog (1 day after treatment); lane 5, positive control; lane 6, negative control. Arrowhead shows the expected band in a positive result.

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References

1. Yamashita J. *Echinococcus* and echinococcosis. In: Morishita K, Komiya Y, Matsubayashi H, editors. Progress of medical parasitology in Japan. Volume 5. Tokyo: Meguro Parasitological Museum. p. 65–123.
2. Doi R, Kanda E, Nihei N, Uchida A. Occurrence of alveolar hydatid disease (multilocular echinococcosis) outside of Hokkaido and a proposal for its prevention. Nippon Koshu Eisei Zasshi. 2000;47:111–26.
3. Eckert J, Conraths FJ, Tackmann K. Echinococcosis: an emerging or re-emerging zoonosis? Int J Parasitol. 2000;30: 1283–94.
4. Kamiya M, Morishima Y, Nonaka N, Oku Y. Epidemiologic surveys of *Echinococcus multilocularis* in the definitive hosts by coproantigen detection between 1996–2000. In: Proceedings of the Joint Meeting of the 70th Annual Meeting of Japanese Society of Parasitology and the 53rd Annual Meeting of the Japan Society of Medical Entomology and Zoology; 2001. Apr 4–6; Yamagata City, Yamagata Prefecture, Japan. Abstract P30W2–1.

5. Morishima Y, Tsukada H, Nonaka N, Oku Y, Kamiya M. Coproantigen survey for *Echinococcus multilocularis* prevalence of red foxes in Hokkaido, Japan. Parasitol Int. 1999;48:121–34.
6. Tsukada H, Morishima Y, Nonaka N, Oku Y, Kamiya M. Preliminary study of the role of red foxes in *Echinococcus multilocularis* transmission in the urban area of Sapporo, Japan. Parasitology. 2000;120:423–8.
7. Doi R, Matsuda H, Uchida A, Kanda E, Kamiya H, Konno K, et al. Possibility of invasion of *Echinococcus multilocularis* into Honshu with pet dogs from Hokkaido and overseas. Nippon Koshu Eisei Zasshi. 2003;50:639–49.
8. Dinkel A, von Nickisch-Rosenegk M, Bilger B, Merli M, Lucius R, Romig T. Detection of *Echinococcus multilocularis* in the definitive host: coprodiagnosis by PCR as an alternative to necropsy. J Clin Microbiol. 1998;36:1871–6.
9. Manfredi MT, Genchi C, Deplazes P, Trevisiol K, Fraquelli C. *Echinococcus multilocularis* infection in red foxes in Italy. Vet Rec. 2002;150:757.
10. Gottstein B, Saucy F, Deplazes P, Reichen J, Demierre G, Busato A, et al. Is high prevalence of *Echinococcus multilocularis* in wild and domestic animals associated with disease incidence in humans? Emerg Infect Dis. 2001;7:408–12.

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New World Hantavirus in Humans, French Guiana

To the Editor: Hantaviruses are etiologic agents for hemorrhagic fever with renal syndrome in Europe and Asia and for hantavirus pulmonary syndrome (HPS) in the Americas. These viruses belong to the family *Bunyaviridae*, genus *Hantavirus*. The natural reservoir of these viruses is wild or domestic rodents. HPS was first described in 1993 in the Four Corners region of the United States (*1*). It is a respiratory illness associated with the inhalation of aerosolized rodent excreta (urine and feces) contaminated with hantavirus particles. Sin Nombre virus (SNV) was the first etiologic agent of this syndrome. Since 1993, HPS has also been reported and confirmed in 6 countries in South America: Argentina, Bolivia, Brazil, Chile, Paraguay, Uruguay (*2*,*3*). Several distinct hantaviruses have been associated with HPS, including Juquituba virus in Brazil (*4*), Andes virus in Southern Argentina (*5*), and Laguna Negra virus in Paraguay (*6*).

French Guiana, an overseas French Administrative Unit in the Amazonian forest complex, is located on the northeastern coast of the South America between Brazil and Suriname. Ninety percent of its surface is tropical rain forest; the remaining 10% is a coastal plain, where 90% of the 200,000 inhabitants live. Cayenne and 2 adjacent towns, Remire and Matoury, constitute the main urban centers, with 80,000 inhabitants, ≈40% of the population. People live mainly in individual houses and small buildings. Many houses are built near forests, except those in the center of Cayenne. The outskirts of Remire and Matoury are surrounded by secondary rain forest, and those of Cayenne by wooded hills, where wild mammals such as rodents live in large numbers.

The prevalence of antibodies to New World hantavirus is unknown in French Guiana. Several cases of atypical pneumonia not linked to other etiologic agents (*Coxiella burnetii*, *Histoplasma boydii*), combined with identification of hantavirus rodent reservoirs in neighboring countries, prompted us to determine the seroprevalence of hantavirus in this area (*7*,*8*).

To estimate the prevalence of antibodies to New World hantavirus, we conducted a retrospective serologic survey of patients with symptoms compatible with HPS. Patients were from all areas of French Guiana: 64% from the urban centers, 7% from rural regions, and 30% from unspecified regions. From April 2002 through April 2004, a total of 420 serum samples were collected from patients with acute-phase febrile illness, unexplained acute respiratory syndrome, or bilateral interstitial pulmonary infiltrates. Diagnosis of Q fever was excluded by negative serologic results for immunoglobulin M (IgM), IgG, or both to *C. burnetii* (bioMérieux, Marcy-l'Etoile, France).

To detect patients with IgG antibodies to SNV, the ELISA described by Feldmann et al. was used (*9*). Briefly, an SNV-positive serum provided by the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) was used as a positive control. Negative controls were obtained by random sampling of all previously negative samples. A sample was considered positive if the net absorbance values (after subtraction of absorbance values with and without antigen) were >0.2 for dilutions of 1:100 and 1:400 and the sum of 4 net absorbance values was >0.95. Seropositive samples were confirmed at CDC.

Antibodies reactive with SNV antigen indicate infection with a New World hantaviruses. However, because SNV is broadly cross-reactive with most New World hantavirus, the specific hantavirus cannot be identified.

The seroprevalence of IgG antibody to hantavirus was 1.42% (6/420) in the selected population. Three other samples showed borderline positivity. Antibody prevalence was not significantly different among the 7 age classes used (0–9, 10–19, 20–29, 30–39, 40–49, 50–59, and >60 years of age, $p = 0.36$, degrees of freedom = 6, by χ^2 test) or by sex ($p = 0.22$, by Fisher exact test).

All patients with seropositive samples lived in the urban centers. The mean age of the 6 patients was 36.0 years (range 24–56 years), and 83% were men. Test results for IgM antibodies to SNV conducted on samples in parallel were negative.

The seroprevalence found in this study was caused by patient exposure to hantavirus. However, in the absence of IgM to SNV, we cannot link the respiratory symptoms observed to recent infection with hantavirus. Lack of information about the patients, especially their clinical history and details of travel to bordering countries, did not permit an association of infection with hantavirus contact in French Guiana. The seroprevalence observed is similar to that in Venezuela, where hantaviruses were isolated from rodents in 1999, but is lower than that observed in regions of Brazil (*10*).

The presence of hantaviruses in neighboring countries, as well as frequent travel by people in and out of French Guiana, has encouraged us to continue studying these viruses. We plan to conduct a study to systematically evaluate hantaviruses by serologic analysis and genomic amplification in persons with suggestive pathology. This study will be carried out in parallel with an investigation of rodent reservoirs of hantaviruses.

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References

1. Nichol ST, Spiropoulou CF, Morzunov S, Rollin PE, Ksiazek TG, Feldmann HA, et al. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science. 1993;262:914–7.
2. Pini N, Levis S, Calderon G, Ramirez J, Bravo D, Lozano E, et al. Hantavirus infection in humans and rodents, northwestern Argentina. Emerg Infect Dis. 2003;9:1070–6.
3. Padula PJ, Colavecchia SB, Martinez VP, Gonzalez Della Valle MO, Edelstein A, Miguel SD, et al. Genetic diversity, distribution, and serological features of hantavirus infection in five countries in South America. J Clin Microbiol. 2000;38: 3029–35.
4. Mattar S, Parra M. Serologic evidence of hantavirus infection in humans, Colombia. Emerg Infect Dis. 2004;10:2263–4.
5. Johnson AM, Bowen MD, Ksiazek TG, Williams RJ, Bryan RT, Mills JN, et al. Laguna Negra virus associated with HPS in western Paraguay and Bolivia. Virology. 1997;238:115–27.
6. Lopez N, Padula P, Rossi C, Miguel S, Edelstein A, Ramirez E, et al. Genetic characterization and phylogeny of Andes virus and variants from Argentina and Chile. Virus Res. 1997;50:77–84.
7. Gardon J, Heraud JM, Laventure S, Ladam A, Capot P, Fouquet E, et al. Suburban transmission of Q fever in French Guiana: evidence of a wild reservoir. J Infect Dis. 2001;184:278–84.
8. Lednicky JA. Hantaviruses: a short review. Arch Pathol Lab Med. 2003;127:30–5.
9. Feldmann H, Sanchez A, Morzunov S, Spiropoulou CF, Rollin PE, Ksiazek TG, et al. Utilization of autopsy RNA for the synthesis of the nucleocapsid antigen of a newly recognized virus associated with hantavirus pulmonary syndrome. Virus Res. 1993;30:351–67.
10. Rivas YJ, Moros Z, Moron D, Uzcategui MG, Duran Z, Pujol FH, et al. The seroprevalences of anti-hantavirus IgG antibodies among selected Venezuelan populations. Ann Trop Med Parasitol. 2003;97:61–7.

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Qinghai-like H5N1 from Domestic Cats, Northern Iraq

To the Editor: Natural infection of several cat species with highly pathogenic avian influenza (HPAI) H5N1 viruses in Thailand (*1–4*) and experimental infection of domestic cats with similar viruses have been reported (*5*,*6*). Thus, literature describing HPAI H5N1 infection of cats is limited to descriptions of infections with a subset of clade I viruses. HPAI H5N1 viruses, highly similar to viruses isolated from Qinghai Lake in western People's Republic of China in spring 2005, are now rapidly disseminating throughout Eurasia and Africa. To our knowledge, this is the first report of a Qinghai-like virus detected in domestic cats. This finding is noteworthy because the host range of influenza viruses is determined by the antigenic characteristics of the hemagglutinin and neuraminidase molecules; clade II viruses are antigenically distinct from clade I viruses, and Qinghai-like viruses are genetically distinct from other clade II viruses.

Personal communications in January 2006 from field veterinarians noted deaths of domestic cats that were associated with suspected (eventually confirmed) H5N1 outbreaks in eastern Turkey (2 villages) and Kurdish northern Iraq (Sarcapcarn in Sulymaniyah Governorate and Grd Jotyar in Erbil Governorate). The clinical conditions of the birds did not suggest HPAI to villagers or consulting veterinarians. In both scenarios in Iraq, results of rapid antigen detection tests with the Anigen kit (Suwon, Republic of Korea), while positive for influenza A, were negative for H5, so the outbreaks were not thought to be caused by HPAI, but concern about the unusual deaths in cats remained.

Because the regions are remote and veterinary services limited, such anecdotal reports have rarely been followed up.

After H5N1 influenza was diagnosed in a person in Sarcapcarn, Kurdish northern Iraq, the government of Iraq requested a World Health Organization investigation, which was supported in part by Naval Medical Research Unit No. 3 veterinarians. While investigating the situation in Erbil Governorate, the team was informed of suspicious deaths in cats associated with the death of all 51 chickens in a household in Grd Jotyar (≈10 km north of Erbil City) From February 3 to February 5, 2006, five cats reportedly died; 2 of these were available for examination on February 8. A sick goose from an adjacent household was killed and underwent necropsy with the cats. Gross pathologic changes in cats were similar to those previously reported, except that severe hemorrhagic pancreatitis was observed (*5*,*6*). Tissues from these animals and archived tissues from 1 of the 51 chickens were conveyed to Cairo for virologic examination.

Table. Detection of influenza A H5 by real-time PCR*

Tissue	Chicken	Goose	Cat 1	Cat 2
Abdominal fluid	ND	+	ND	ND
Blood	ND	–	–	ND
Heart	+	ND	ND	ND
Trachea	ND	–	+	+
Lung	+	+	+	+
Kidney	ND	ND	–	ND
Spleen	ND	–	–	–
Pancreas	ND	ND	+	+
Lymph node	ND	ND	–	ND
Liver	+	ND	–	+
Proventriculus	+	ND	N/A	N/A
Small intestine	+	+	–	ND
Large intestine	ND	–	+	+
Cecum	ND	+	ND	ND
Feces	ND	ND	+	ND

*ND, not done. Samples were tested by real time PCR for influenza A (matrix protein) and if positive, for H5 (*7*). All samples denoted as positive were positive for both influenza A and H5. Chicken samples were obtained previously by local veterinarians based on their sampling protocols. Goose and cat samples were obtained by S. Felt; only grossly abnormal tissues were sampled.

An influenza A H5 virus was present in multiple organs in all species from the outbreak site in Grd Jotyar (Table). cDNA for sequencing was amplified directly from RNA extracts from pathologic materials without virus isolation. On the basis of sequence analysis of the full HA1 gene and 219 amino acids of the HA2 gene, the viruses from the goose and 1 cat from Grd Jotyar and from the person who died from Sarcapcarn (sequence derived from PCR amplification from first-passage egg material) are >99% identical at the nucleotide and amino acid levels (GenBank nos. DQ435200–02). Thus, no indication of virus adaptation to cats was found. The viruses from Iraq are most closely related to currently circulating Qinghai-like viruses, but when compared with A/bar-headed goose/Qinghai/65/2005 (H5N1) (GenBank no. DQ095622), they share only 97.4% identity at the nucleic acid level with 3 amino acid substitutions of unknown significance. On the other hand, the virus from the cat is only 93.4% identical to A/tiger/Thailand/CU-T4/2004 (H5N1) (GenBank no. AY972539). These results are not surprising, given that these strains are representative of different clades (*8*,*9*). Sequencing of 1,349 bp of the N gene from cat 1 and the goose (to be submitted to GenBank) show identity at the amino acid level, and that the N genes of viruses infecting the cat and goose are >99% identical to that of A/bar-headed goose/Qinghai/65/2005(H5N1). These findings support the notion that cats may be broadly susceptible to circulating H5N1 viruses and thus may play a role in reassortment, antigenic drift, and transmission.

The route of infection in these cats cannot be determined definitively. How cats behave when eating birds makes both oral and respiratory infection possible. However, the source of infection seems clear in that an identical H5N1 virus was detected in samples from a goose from the same dwelling, and an H5 virus was detected from archived samples from 1 of 51 chickens that died over the course of a few days. The potential for horizontal spread cannot be ruled out since we detected viral RNA in gut, stool, and trachea; clinical signs developed in all cats, and all died from the acute illness 2–4 days after the chicken deaths began; therefore, simultaneous exposure seems likely. Death in cats, spatially and temporally associated with unusual deaths in poultry, especially when the cats show positive results of a rapid antigen detection test for influenza A, should be considered to indicate a presumptive diagnosis of HPAI, and appropriate response should ensue.

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References

1. Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, et al. Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis. 2004;10:2189–91.
2. Thanawongnuwech R, Amonsin A, Tantilertcharoen R, Damrongwatanapokin S, Theamboonlers A, Payungporn S, et al. Probable tiger-to-tiger transmission of avian influenza H5N1. Emerg Infect Dis. 2005;5:699–701. Erratum in Emerg Infect Dis. 2005;11:976.
3. Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N, et al. Avian influenza H5N1 in naturally infected domestic cat. Emerg Infect Dis. 2006;12: 681–3.
4. Amonsin A, Payungporn S, Theamboonlers A, Thanawongnuwech R, Suradhat S, Pariyothorn N, et al. Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. Virology. 2006;344:480–91.
5. Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA, et al. Influenza A virus (H5N1) Infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol. 2006;168:176–83.
6. Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, Fouchier R, et al. Avian H5N1 influenza in cats. Science. 2004;306:241.
7. Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Prdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 2002; 40:3256–60.
8. World Health Organization Global Influenza Program Surveillance Network. Evolution of H5N1 avian influenza viruses in Asia. Emerg Infect Dis. 2005;11: 1515–21.
9. Chen H, Smith GJD. LI KS, Wang J, Fan XH, Rayner JM, et al. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci U S A. 2006;103:2845–50.

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Classifying *Escherichia coli*

To the Editor: Enteropathogenic *Escherichia coli* (EPEC), 1 of the 6 pathotypes of diarrheogenic *E. coli* (DEC), promotes attaching-effacing lesions in eukaryotic cells. These lesions are mediated by intimin, an outer membrane adhesive protein encoded by the *eae* (*E. coli* attaching-effacing) gene (*1*). EPEC is currently subdivided into typical and atypical subgroups. While typical EPEC carry the EPEC adherence factor plasmid (pEAF) that encodes the bundle-forming pilus (BFP) and a complex regulator of various virulence genes (Per) (*1*), atypical EPEC is devoid of pEAF (or does not express a functional BFP) (*1*,*2*). Typical EPEC expresses the localized pattern of adherence (LA), which is characterized by compact bacterial clusters on HeLa and HEp-2 cells (*1*). Conversely, atypical EPEC most often expresses the LA-like pattern (with loose bacterial clusters) or adherence patterns of other DEC pathotypes (*2*).

Enteroaggregative *E. coli* (EAEC), another DEC pathotype, is identified by the characteristic aggregative pattern of adherence (AA) in HeLa/HEp-2 cells; bacteria attach in aggregates to cell surfaces as well as around cells (*1*,*3*). EAEC colonizes the intestinal mucosa, forming a thick biofilm that favors prolonged colonization and induces malnutrition (*1*–*3*). Actually, this pathotype is heterogeneous regarding the presence of putative virulence genes and has recently been subgrouped into typical and atypical EAEC, which carry and lack AggR (a global regulator of EAEC virulence), respectively (*1*,*3*).

We recently conducted a study at the Instituto de Puericultura e Pediatria Martagão Gesteria in Rio de Janeiro, Brazil, on the etiology of diarrhea affecting children of low socioeconomic status (V.B.C. Girão et al., unpub. data). In the study, all *E. coli* isolates were analyzed regarding their adherence patterns in HeLa cells and the presence of specific virulence genes of the DEC pathotypes, according to previously reported methods (*4*,*5*). Among 481 children (<2 years old) with diarrhea who were examined, 16 (3.3%) carried *E. coli* strains that co-expressed LA and AA (LA/AA), a phenotype not found among strains of 99 control children without diarrhea at the same hospital. The LA/AA phenotype was confirmed in individual colonies of each strain as well as in HEp-2 cells. In both cell lineages, prolonged assays (6 hours) showed that a mature biofilm that masked the LA phenotype had developed.

Although LA/AA co-expression in some human *E. coli* has been previously reported by Bouzari et al. (*6*), further information on these isolates is lacking. Moreover, since the expression of LA and AA is used to classify fecal *E. coli* as typical EPEC and EAEC (*1*,*3*), respectively, the classification of such strains within the DEC pathotypes is difficult. To determine their most appropriate classification, we further characterized the 16 LA/AA strains of our collection (Table). Colony hybridization assays used to search for additional *E. coli* virulence genes (*bfpA*, *perA*, E-*hly*, *daaC*, *cdt*, *cnf*, *hly*, *aggR*, *aggC*, *aafC*, *aap*, *shf*, *irp2*, *pet*, *pic*, *astA*, *pap*, *afa*, *sfa*, *efa*, *paa*, *saa*, *enfA*) (*1*,*3*–*5*,*7*) showed that all strains carried *eae*, *bfpA*, and *perA*, and 13 also carried the EAF sequence (a cryptic pEAF marker). Less commonly found genes were *paa*, *shf*, *irp*2, *astA*, and *efa*, and the remaining genes were absent. BFP expression was confirmed in all strains by immunoblot, and positivity in the fluorescent actin staining assay (*8*) demonstrated that they can produce attaching/effacing lesions. PCR analysis of 4 (α, β, γ, and δ) (*9*) of at least 10 recognized intimin subtypes (*1*) showed that subtype δ was the most frequent. Serotyping (*5*) identi-

Table. Genotypic and phenotypic properties of 16 *Escherichia coli* strains that co-express localized and aggregative patterns of adherence*

Strain	Virulence genes	Intimin subtype	Serotype
98180	*eae*, *bfp*A, EAF, *per*A, *paa*	δ	O2:H45
99137	*eae*, *bfp*A, EAF, *per*A, *paa*	δ	O2:H45
21153	*eae*, *bfp*A, EAF, *per*A, *shf*	δ γ†	O55:H51
21187	*eae*, *bfp*A, EAF, *per*A, *shf*	δ γ†	O55:H51
99253	*eae*, *bfp*A, EAF, *per*A, *shf*	δ γ†	O55:HNM
99329	*eae*, *bfp*A, EAF, *per*A, *shf*	δ γ†	O55:HNM
99197	*eae*, *bfp*A, EAF, *per*A	β δ†	O119:H6
98288	*eae*, *bfp*A, EAF, *per*A, *irp2*	β	O119:H6
22622	*eae*, *bfp*A, EAF, *per*A, *irp2*	β	O119:H6
99336	*eae*, *bfp*A, *per*A, *paa*, *astA*, *efa*	α	O142:H6
98351	*eae*, *bfp*A, EAF, *per*A, *astA*	γ	O145:H45
22652	*eae*, *bfp*A, EAF, *per*A, *irp2*	(–)	O178:H33
99245	*eae*, *bfp*A, *per*A, *paa*, *efa*, *ast*A	α	ONT:H6
98025	*eae*, *bfp*A, *per*A, *paa*	α	ONT:H6
98366	*eae*, *bfp*A, EAF, *per*A, *efa*	β	ONT:H7
22150	*eae*, *bfp*A, EAF, *per*A, *astA*	α	ONT:H10

*Isolated from children with diarrhea. EAF, enteropathogenic *E. coli* adherence factor; –, nontypable with the primers tested.
†Intimin types undetermined because amplification products of the expected size were obtained with 2 intimin pairs of primers.

fied at least 10 distinct serotypes among the 16 strains, which demonstrated that they do not make up a single clone. Two serotypes (O119:H6 and O142:H6) are commonly found among typical EPEC (*2*). Certain typical and atypical EPEC serotypes have been associated with distinct intimin subtypes (*9*). Likewise, our LA/AA strains of the same serotype carried the same intimin subtype. Recently, Carvalho et al. (*10*) detected LA/AA expression in 4 of 21 *eae*-positive *E. coli* strains isolated from monkeys with diarrhea. All 4 strains expressed BFP and lacked the EAF sequence; as in our study, 1 belonged to serotype O142:H6 and carried intimin α.

E. coli classification within the DEC pathotypes has epidemiologic and clinical implications for managing diarrheal diseases. However, finding *E. coli* isolates that co-express LA/AA reiterates the difficulty of assigning bacteria to groups on the basis of their adherence phenotype or genotype (particularly when based on mobile genetic elements). Since our analysis with molecular methods showed that these strains carry more characteristics of typical EPEC and lack the AggR regulon, we propose that they be classified as typical EPEC. Typical EPEC are recognized as pathogens, whereas atypical EAEC are not (*3*). In addition, the ability to simultaneously induce attaching/effacing lesions and biofilm production may increase the potential of these strains to cause diarrhea and prolong bacterial residence in the intestines, thus worsening malnutrition in the patient.

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References

1. Kaper JB, Nataro JP, Mobley HLT. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2:123–40.
2. Trabulsi LR, Keller R, Gomes TAT Typical and atypical enteropathogenic *Escherichia coli*. Emerg Infect Dis. 2002;8:508–13.
3. Nataro JP. Enteroaggregative *Escherichia coli* pathogenesis. Curr Opin Gastroenterol. 2005;21:4–8.
4. Vieira MAM, Andrade JRC, Trabulsi LR, Rosa ACP, Dias AMG, Ramos SRTS, et al. Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry *eae* and lack the EPEC adherence factor and shiga toxin DNA probe sequences. J Infect Dis. 2001;183:762–72.
5. Gomes TAT, Irino K, Girão DM, Girão VBC, Guth BEC, Vaz TMI, et al. Emerging enteropathogenic *Escherichia coli* strains? Emerg Infect Dis. 2004;10:1851–5.
6. Bouzari S, Jafari A, Farhoudi-Moghaddam AA, Shokouhi F, Parsi M. Adherence of non-enteropathogenic *Escherichia coli* to HeLa cells. J Med Microbiol. 1994;40:95–7.
7. Monteiro-Neto V, Bando SY, Moreira-Filho CA, Girón JA. Characterization of an outer membrane protein associated with haemagglutination and adhesive properties of enteroaggregative *Escherichia coli* O111:H12. Cell Microbiol. 2003;5:533–47.
8. Knutton S, Baldwin T, Williams PH, McNeish AS. Actin accumulation at sites of bacterial adhesion to tissue cultures cells: basis of a new diagnostic test for enteropathogenic and enterohaemorragic *Escherichia coli*. Infect Immun. 1989;57:1290–8.

9. Adu-Bobie J, Frankel G, Bain C, Gonçalves AG, Trabulsi LR, Douce G, et al. Detection of intimin alpha, beta, gamma, and delta, four intimin derivatives expressed by attaching and effacing microbial pathogens. J Clin Microbiol. 1998;36:662–8.
10. Carvalho VM, Gyles CL, Ziebell K, Ribeiro MA, Catão-Dias JL, Sinhorini IL, et al. Characterization of monkey enteropathogenic *Escherichia coli* (EPEC) and human typical and atypical EPEC serotype isolates from neotropical nonhuman primates. J Clin Microbiol. 2003;41:1225–34.

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Toscana Virus RNA in *Sergentomyia minuta* Flies

To the Editor: Toscana virus (TOSV) (family *Bunyaviridae*, genus *Phlebovirus*) is an arthropodborne virus transmitted by sandflies. Reports of infections in travelers, clinical research, and epidemiologic studies have shown that TOSV affects the central nervous system and is a major cause of meningitis and encephalitis in Mediterranean countries in which the virus circulates (*1*). In central Italy, this virus is the primary cause of meningitis from May to October, far exceeding enteroviruses as a cause of disease (*2*). In other northern Mediterranean countries, TOSV is among the 3 most prevalent viruses associated with meningitis during the warm seasons (*1*). TOSV has recently been associated with human disease in France (*3*,*4*) and was originally isolated in Italy from *Phlebotomus perniciosus*, then from *P. perfiliewi*, but never from *P. papatasi*. TOSV has also been isolated from the brain of a bat in areas where *P. perniciosus* and *P. perfiliewi* were present, but no hemagglutination-inhibiting antibodies were found in sera from these bats (*5*). In Spain, 2 isolates of TOSV were recovered from 103 pools of sandflies; sequence analyses showed that they were genetically divergent from the Italian strains (*6*). To date, TOSV had not been isolated from sandflies collected in France.

In July 2005, a total of 123 *Sergentomyia minuta* were collected in a 4-day period near Marseille, southeastern France. This work was part of a larger collaborative study, the results of which will be published separately. CDC miniature light traps (John W. Hock Co., Gainesville, FL, USA) were adapted to sandflies with an ultra-fine mesh. Traps were hung 1–2 m above the ground. They were placed in the late afternoon inside or near animal housing facilities (for chickens, rabbits, goats, or horses) in the suburbs of Marseille for 4 successive nights. In these areas, large numbers of geckos were noticed. Each morning, sandflies were collected, identified morphologically, and placed in 1.5-mL Eppendorf tubes. *S. minuta* flies were identified by appearance, and genus was confirmed by sequence analysis, as previously reported (*7*).

Five pools of the captured *S. minuta* were prepared with a maximum of 30 flies per pool. They were ground in 20% fetal bovine serum–enriched phosphate-buffered saline in a Mixer Mill MM300 (Qiagen, Courtaboeuf, France) with one 3-mm tungsten bead and clarified by low-speed centrifugation. We used 200 µL supernatant for total RNA purification onto the MagNAPure platform with the MagNA Pure LC RNA High Performance Kit (Roche Diagnostics, Meylan, France). We used 10 µL RNA suspension for reverse transcription PCR, with primers targeting either a consensus sequence for the phlebovirus polymerase gene (L RNA segment) or Toscana virus (*8*) and the nucleoprotein (N) gene (S RNA segment) specifically (*9*).

Only 1 TOSV-positive pool was observed with primers specific to TOSV polymerase and N genes, respectively. A positive result was observed with primers NPhlebo2+ and ATos2, previously found to target polymerase genes of a range of phleboviruses (*8*). This result was confirmed by sequence analysis (GenBank accession no. DQ195277), which showed 82.8% and 96% identity at the nucleotide and amino acid level, respectively, with a TOSV isolate from Italy (GenBank accession no. X68414). The same pool also tested positive with primers (5′-CGTRGCAGCCACYTCATTAG-3′ and 5′-GTGTCGGCYGCSTTTG-TTCC-3′) designed in this study from the alignment of the 13 sequences of TOSV retrieved from GenBank (accession nos. are shown in the Figure). Comparing the sequence of this 272-bp PCR product with homologous sequences of selected phleboviruses available in the GenBank database showed 97.4%, 87.1%–88.2%, and 78.7% identity at the nucleotide level with TOSV strains isolated in Italy, TOSV isolated in Spain, and sandfly fever Naples virus (Sabin strain), respectively. Phylogenetic analyses of the N gene indicated that this virus clustered with TOSV strains circulating in Italy and Spain but is most closely related to isolates from Italy (Figure). Comparative analysis within the polymerase gene confirmed these data, but distance analysis with sequences of Spanish TOSV was not possible because genetic data were lacking in public databases. The remaining 400-µL volume of sandfly material was used to attempt virus isolation in Vero cells and by intracerebral injection of

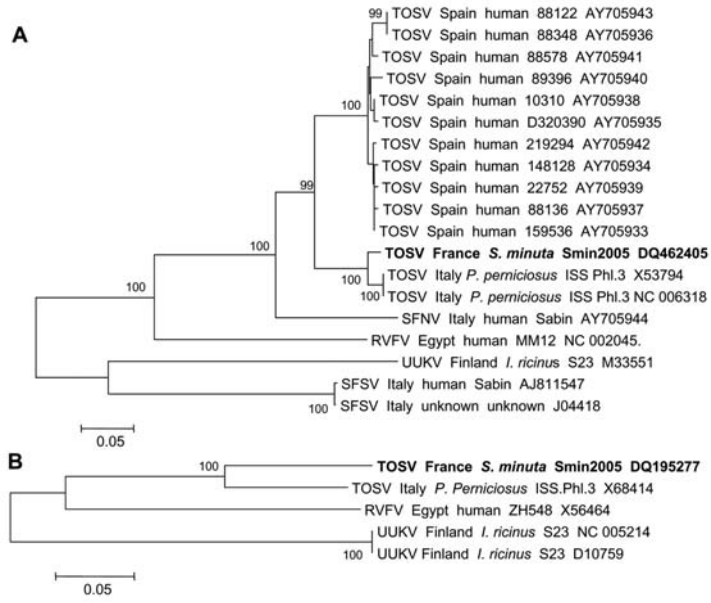

Figure. Phylogenetic analysis of Toscana virus (TOSV) from *Sergentomyia minuta* based on 272- and 128-nucleotide sequences in the nucleoprotein (A) and polymerase (B) genes of viral RNA, respectively. Distances and groupings were determined by the p-distance algorithm and neighbor-joining method with the MEGA software program. Bootstrap values >95% are indicated and correspond to 500 replications. For each sequence, the following information is indicated: virus/geography/host/isolate name/GenBank accession no. SFSV, sandfly fever Sicilian virus; SFNV, sandfly fever Naples virus; RVFV, Rift Valley fever virus; UUKV, Uukuniemi virus. Sequences determined in this study are shown in **boldface**.

2-day-old suckling mice, but no virus was recovered.

To our knowledge, this is the first time that TOSV has been detected in phlebotomine flies other than *P. perniciosus* and *P. perfiliewi*. *S. minuta* was identified with morphologic keys and confirmed by sequencing a portion of the 28S gene (*7*). *Sergentomyia* spp. have been reported to be infected by a variety of different RNA viruses, such as Chandipura (*10*), Saboya (*11*), Tete, and 2 unclassified viruses (ArD 95737 and ArD 111740). However, *S. minuta* feed on reptiles but not on humans, which may prevent them from being vectors of human infection. Additional studies are needed to better understand the role of *Sergentomyia* spp. and other arthropods in the ecology of TOSV. Whether TOSV also circulates in *Phlebotomus* spp. in France remains to be determined, but the evidence for human infections with this virus shows that more extensive investigations are needed to understand the role of this arbovirus in neurologic diseases in the Mediterranean.

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References

1. Charrel RN, Gallian P, Navarro-Mari J-M, Nicoletti L, Papa A, Sanchez-Seco MP, et al. Emergence of Toscana virus in Europe. Emerg Infect Dis. 2005;11:1657–63.
2. Valassina M, Meacci F, Valensin PE, Cusi MG. Detection of neurotropic viruses circulating in Tuscany: the incisive role of Toscana virus. J Med Virol. 2000;60:86–90.
3. Hemmersbach-Miller M, Parola P, Charrel RN, Paul Durand J, Brouqui P. Sandfly fever due to Toscana virus: an emerging infection in southern France. Eur J Intern Med. 2004;15:316–7.
4. Peyrefitte CN, Devetakov I, Pastorino B, Villeneuve L, Bessaud M, Stolidi P, et al. Toscana virus and acute meningitis, France. Emerg Infect Dis. 2005;11:778–80.
5. Verani P, Ciufolini MG, Caciolli S, Renzi A, Nicoletti L, Sabatinelli G, et al. Ecology of viruses isolated from sand flies in Italy and characterization of a new *Phlebovirus* (Arbia virus). Am J Trop Med Hyg. 1988;38:433–9.
6. Sanbonmatsu-Gámez S, Pérez-Ruiz M, Collao X, Sánchez-Seco MP, Morillas-Márquez F, de la Rosa-Fraile M, et al. Toscana virus in Spain. Emerg Infect Dis. 2005;11:1701–7.
7. Depaquit J, Perrotey S, Lecointre G, Tillier A, Tillier S, Ferte H, et al. Molecular systematics of *Phlebotominae*: a pilot study. Paraphyly of the genus *Phlebotomus*. C R Acad Sci III. 1998;321:849–55.
8. Sánchez Seco MP, Echevarría JM, Hernández L, Estévez D, Navarro Marí JM, Tenorio A. Detection and identification of Toscana and other phleboviruses by RT-PCR assays with degenerated primers. J Med Virol. 2003;71:140–9.
9. Valassina M, Cusi MG, Valensin PE. Rapid identification of Toscana virus by nested PCR during an outbreak in the Siena area of Italy. J Clin Microbiol. 1996;34:2500–2.
10. Geevarghese G, Arankalle VA, Jadi R, Kanojia PC, Joshi MV, Mishra AC. Detection of Chandipura virus from sand flies in the genus *Sergentomyia* (Diptera: Phlebotomidae) at Karimnagar District, Andhra Pradesh, India. J Med Entomol. 2005;42:495–6.
11. Ba Y, Trouillet J, Thonnon J, Fontenille D. *Phlebotomus* of Senegal: survey of the fauna in the region of Kedougou. Isolation of arbovirus. Bull Soc Pathol Exot. 1999;92:131–5.

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Rat-bite Fever, Canada

To the Editor: Rat-bite fever was once considered an infection exclusive to children living in poverty; however, dense urban housing and changing pet-keeping practices may be altering this profile (*1*,*2*). To date, ≈200 cases of rat-bite fever have been reported in the United States (*3*), and a recent study reported a 2-fold increased incidence in California during the 1990s (*1*). We report on 2 cases that occurred in Ontario, Canada, in the early 2000s.

The first case occurred in a previously healthy 29-year-old man who was bitten on the finger by a pet rat. The wound healed spontaneously. After 24 hours, fever and emesis developed; 4 days later, diffuse maculopapular rash and migratory arthritis of the knees, ankles, and finger joints ensued. Physical examination showed a maculopapular rash over the lower extremities, an effusion of the left knee, and a warm, erythematous left ankle.

Laboratory investigations showed hemoglobin level of 134 g/L, leukocyte count of 16.0×10^9/L, and neutrophil count of 13.8×10^9/L. Aspiration of the knee produced 70 cm^3 of cloudy fluid; synovial fluid analysis showed 666×10^6/L leukocytes with a predominance of neutrophils.

Ceftriaxone, 2 g once a day, was given intravenously for 7 days. Although symptoms improved within 24 hours, the effusion recurred within 48 hours of discontinuing the initial course of ceftriaxone. The knee was surgically drained, and ceftriaxone was continued for 5 weeks. Systemic symptoms and the effusion resolved.

The second case occurred in a previously healthy 9-year-old girl who had mucosal contact with a pet rat. She sought treatment after 7 days of generalized maculopapular and pustular rash and 10 days of fever and headache. She had an associated asymmetric, migratory arthritis.

Physical examination showed superficial scratches from the rat; temperature of 39.6°C; heart rate of 102 bpm; swelling, erythema, and decreased range of motion in several joints; and pustular lesions on the soles of the feet.

The patient's leukocyte count was 8.3×10^9/L. Synovial fluid from the knee showed 45.5×10^6/L leukocytes with 89% neutrophils; the culture showed no growth. Gram stains of blood and pustule swabs showed large, pleomorphic, gram-negative bacilli with long filaments and irregular swellings. Growth occurred on the blood culture after 28 hours of aerobic incubation at 35°C in 10% horse serum. Characteristic puff-ball colonies of *Streptobacillus moniliformis* were seen in supplemented thioglycolate broth.

Identification of the organism was confirmed by using the Sherlock (MIDI Inc., Newark, DE, USA) system. The major cellular fatty acid components of the isolate matched an *S. moniliformis* reference strain. The patient received penicillin and gentamicin intravenously for 6 days and was discharged home with a 10-day regimen of amoxicillin. One year later, she remained asymptomatic.

Rat-bite fever commonly results from infection with the zoonotic pathogens *S. moniliformis* and *Spirillum minus*. *S. moniliformis* is more common in Western countries, and *S. minus* predominates in Asia (*3*). *S. moniliformis* colonizes the nasopharynx of healthy rats (*4*) and is transmitted by the bite or scratch of rats, squirrels, mice, guinea pigs, and, rarely, cats and other rodent predators. Occasionally, it is transmitted by ingestion of contaminated milk or water. (*5*,*6*). The site of inoculation with *S. moniliformis* usually heals before systemic symptoms develop. After the incubation period of 1 to 22 days, patients experience fever, chills, myalgia, headache, and rash. The rash consists of macules, vesicles, and pustules on the extremities; soles and palms are frequently involved. Joint symptoms range from polyarthralgia to migratory polyarthritis with purulent effusions. A nonsuppurative migratory polyarthritis occurs in ≈50% of patients (*5*,*7*). In rare cases, rash and arthritis may be absent (*8*).

When *S. minus* (a spirochete) is introduced by rat bite, the bite wound initially heals but then ulcerates, followed by regional lymphadenopathy and a distinctive rash of red and purple plaques. Arthritic symptoms are rare (*9*).

Complications of rat-bite fever include destructive joint disease, pericarditis, endocarditis, abscesses, pneumonia, parotitis, pancreatitis, and, rarely, meningitis and amnionitis. Development of endocarditis results in a mortality rate of up to 50% (*5*).

S. moniliformis can be isolated and cultured from synovial fluid, blood, and abscesses. By contrast, *S. minus* has not been recovered on artificial media but can be seen by using darkfield microscopy with Giemsa or Wright stains.

Laboratory personnel must be notified when rat-bite fever is suspected because *S. moniliformis* does not grow in a routine sheep blood or MacConkey agar; it requires rat or horse serum, defibrinated blood, or ascitic fluid to sustain growth. Growth of *S. moniliformis* is inhibited by sodium polyanetholesulfonate, a substance that is added to blood culture bottles to inhibit the antimicrobial action of blood (*4*,*8*).

Optimal treatment for rat-bite fever is penicillin G given intravenously for 7 to 10 days, followed by penicillin V taken orally for 7 days. Alternatively, tetracycline may be used (*5*,*7*,*9*).

Although rat-bite fever is uncommon, it is increasingly seen as a result of changing patterns of urban living and pet-keeping practices. If unrecognized, this infection can have

debilitating sequelae and can be life threatening.

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References

1. Graves MH, Janda MJ. Rat-bite fever (*Streptobacillus moniliformis*): a potential emerging disease. Int J Infect Dis. 2001;5: 151–4.
2. Morse SS. Factors in the emergence of infectious disease. Emerg Infect Dis. 1995;1:7–15.
3. Freels LK, Elliott SP. Rat bite fever: three case reports and a literature review. Clin Pediatr (Phila). 2004;43:291–5.
4. Salmon RL, McEvoy MB. Rat-bite fevers. In: Zoonosis. Palmer SR, Soulsby S, Simpson DIH, editors. Toronto: Oxford Medical Publications; 1998. p. 187–90.
5. Hambridge SJ, Ogle JW. Index of suspicion. Case 1. Diagnosis: rat-bite fever. Pediatr Rev. 2001;22:95–103.
6. Parker F Jr, Hudson HP. The etiology of Haverhill fever (erythema arthriticum epidemicum). Am J Pathol. 1926;2:357–79.
7. Washburn R. *Streptobacillus moniliformis* (Rat-bite fever). In: Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. 5th ed. Toronto: Churchill Livingstone Inc.; 2000. p. 2422–4.
8. Lambe DW Jr, McPhedran AM, Mertz JA, Stewart P. *Streptobacillus moniliformis* isolated from a case of Haverhill fever: biochemical characterization and inhibitory effect of sodium polyanethol sulfonate. Am J Clin Pathol. 1973;60:854–60.
9. Committee on Infectious Diseases, American Academy of Pediatrics. 2003 Red book: Report of the Committee on Infectious Diseases. 26th ed. Elk Grove Village, IL: The American Academy of Pediatrics; 2000. p. 482–3.

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Cutaneous Injury and *Vibrio vulnificus* Infection

To the Editor: *Vibrio vulnificus* infection is transmitted by eating contaminated seafood or by exposure to seawater through an open wound (*1*). Among immunocompromised persons, especially those with chronic liver disease, *V. vulnificus* can cause a life-threatening illness characterized by blistering skin lesions, necrotizing fasciitis, and septic shock (*2–5*). However, the epidemiology and risk factors for severe forms of *V. vulnificus* infection among healthy persons are less well documented (*4–6*).

We conducted a retrospective clinical record review of *V. vulnificus* infections in persons admitted to all public hospitals from January 1, 2003, through August 31, 2005, in Hong Kong, which has a population of >6 million persons. We defined a case-patient as a patient with culture of *V. vulnificus* from any clinical specimen. A record search of clinical case notes was performed through a computerized clinical management system maintained by the Hospital Authority, which manages all public hospitals in Hong Kong. For each case-patient identified, we reviewed demographic data (age, sex, occupation, residence), clinical and laboratory data (date of onset, symptoms, laboratory investigation findings, diagnosis, outcome), and potential risk factors (past health and possible source of exposure) associated with the case. We compared previously healthy patients with patients who had predisposing medical conditions in terms of demographic profile, clinical signs and symptoms and outcome, and known exposure factors. Mann-Whitney U tests, χ^2 tests, or Fisher exact tests were used to detect significant differences ($\alpha = 0.05$).

We identified 29 cases over the 32-month study period. Twenty-two (76%) patients had disease onset from May through August, the summer season in Hong Kong. Fifteen (52%) cases were in men, and the median age was 70 years (range 24–82 years). Fifteen (52%) patients had underlying illnesses that were known to predispose them to *V. vulnificus* infection, including chronic liver disease (30%), chronic renal failure (15%), diabetes mellitus (7%), and thalassemia major (3%). Fourteen (48%) patients were previously healthy. No significant differences in age and sex were found.

Among the 14 previously healthy patients, the consequences of *V. vulnificus* infection included necrotizing fasciitis (70%), severe cellulitis (7%), primary septicemia (14%), and gastroenteritis (7%). Two patients who had necrotizing fasciitis and 1 patient with primary septicemia died. Compared with patients with predisposing medical conditions, patients with a history of good health had a higher (but not significant) proportion of necrotizing fasciitis (70% vs 47%, $p = 0.12$), a lower proportion of septicemia (14% vs. 27%, $p = 0.26$), and an equal number of severe cases of cellulitis (7% vs. 7%). Furthermore, fewer patients with a history of good health died than did patients with predisposing illnesses (21% vs. 33%, $p = 0.25$). The median duration between symptom onset and admission for all patients was 1 day (range 0–3 days), with no significant difference between the 2 groups.

A history of cutaneous injury or a skin prick from a seafood part (e.g., fish fin, shrimp spine, or crab leg) was significantly more common among previously healthy patients than among patients with predisposing illnesses (70% vs. 27%, $p = 0.02$). Ten (83%) of the 12 previously healthy patients with necrotizing fasciitis and septicemia reported a history of cutaneous injury. The corresponding proportion was significantly lower (31%) among patients with predisposing medical conditions ($p = 0.01$). Among

all 29 patients, a history of eating raw oysters or other raw or undercooked seafood before illness onset was uncommon and was only reported by 1 patient. Although *V. vulnificus* has not been proven as the cause of gastroenteritis, Hseuh et al. have suggested that such results might have occurred because patients with diarrhea seldom sought care from a large teaching hospital or saved stool samples for investigation (*7*).

V. vulnificus infection was first reported in humans in 1979 (*1*). Since then, most case reports have focused on immunocompromised persons and their risk from eating raw oysters among (*4–6*). Our study found that a considerable proportion of *V. vulnificus* infections in Hong Kong occur among healthy persons. Furthermore, severe forms of the infection, such as necrotizing fasciitis and septicemia, are relatively common among healthy persons, although they may cause fewer deaths than they do among persons with predisposing medical conditions. Among healthy persons, *V. vulnificus* infection is most likely associated with a history of cutaneous injury caused by handling seafood, which can allow the bacteria to enter the body through an open wound. The risk of exposure is more important in this locality than in other areas where swimming or eating raw oysters and undercooked seafood are the major risk factors (*4,6–8*), possibly because fresh seafood is widely consumed, and seafood is easily accessible in wet markets in Hong Kong. Our study shows that the risk is higher during the summer, which is consistent with the fact that *V. vulnificus* is more active in warmer temperatures (*9*). We suggest that all persons, even healthy persons, exercise caution to avoid injury while handling seafood. Physicians should consider possible *V. vulnificus* infection when diagnosing a rapidly progressive skin and soft tissue infection in a healthy person who reports an injury from handling seafood.

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References

1. Blake PA, Merson MH, Weaver RE, Hollis DG, Heublein PC. Disease caused by a marine *Vibrio*. Clinical characteristics and epidemiology. N Engl J Med. 1979;300:1–5.
2. Klontz KC, Lieb S, Schreiber M, Janowski H, Baldy L, Gunn RA. Syndromes of *Vibrio vulnificus* infections: clinical and epidemiologic features in Florida cases, 1981–1987. Ann Intern Med. 1988;109:318–23.
3. Mitra AK. *Vibrio vulnificus* infection: epidemiology, clinical presentation, and prevention. South Med J. 2004;97:118–9.
4. Gholami P, Lew SQ, Klontz KC. Raw shellfish consumption among renal disease patients. A risk factor for severe *Vibrio vulnificus* infection. Am J Prev Med. 1998;15:243–5.
5. Haq SM, Dayal HH. Chronic liver disease and consumption of raw oysters: a potentially lethal combination—a review of *Vibrio vulnificus* septicemia. Am J Gastroenterol. 2005;100:1195–9.
6. Potasman I, Paz A, Odeh M. Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. Clin Infect Dis. 2002;35:921–8.
7. Hsueh PR, Lin CY, Tang HJ, Lee HC, Liu JW, Liu YC, et al. *Vibrio vulnificus* in Taiwan. Emerg Infect Dis. 2004;10:1363–8.
8. Tacket CO, Brenner F, Blake PA. Clinical features and an epidemiological study of *Vibrio vulnificus* infections. J Infect Dis. 1984;149:558–61.
9. Shapiro R, Altekruse S, Hutwagner S, Bishop R, Hammond R, Wilson S, et al. The role of Gulf Coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988–1996. J Infect Dis. 1998;178:752–9.

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Neorickettsia helminthoeca in Dog, Brazil

To the Editor: *Neorickettsia helminthoeca* causes salmon poisoning disease (SPD) in canids. SPD has been described only in the United States and the northwestern Pacific region of Canada (*1*). This report complements previous pathologic findings (*2*) and identifies SPD beyond the known disease-endemic region.

From 2001 to 2005, 20 dogs (5 mongrels and 15 beagles) showed pathologic lesions consistent with SPD. All beagles were born in coastal Florianópolis, Santa Catarina, Brazil, and later transferred to Maringá, Paraná, Brazil, for the last 3–4 years of life. Lymph nodes, spleen, liver, and intestines from 10 beagles were aseptically obtained at necropsy in Maringá and frozen at −20°C until used at the Johns Hopkins Medical Institutions in Baltimore, Maryland.

Genomic DNA was extracted from frozen tissues with QIAamp DNA Mini Kits (Qiagen, Valencia, CA, USA). DNA from *N. helminthoeca* and *Anaplasma phagocytophilum* was used as a positive control. Nuclease-free water was used as a negative control. We used gene-specific primers for *Neorickettsia* spp. 16S rRNA (*rrs*) (NeoSH-F; 5′-TAGGCCCGCGTTATGATTAGCTTGT-3′ and NeoSH-R; 5′-TACAACCCAAGGGCCTTCATCACT-3′) and *N. helminthoeca* RNA polymerase β-subunit (*rpo*B) (NH-rpoB-F: 5′-TGTCTTCGAAGGCC-

CAAAGACAGA-3′ and NH-rpoB-R: 5′-AGAACCGATAGAGCGGGCATGAAT-3′) (*3*) and heat-shock protein *gro*ESL (NH-groESL-F: 5′-AGGCTACTTCGCAGGCAAATGAGA-3′ and NH-groESL-R: 5′-CACGCTTCATTCCGCCCTTTAACT-3′) (*4*,*5*). Citrate synthase (*glt*A) gene primers (*6*) were also used. Two PCRs were conducted to maximize sensitivity.

Specificity of *N. helminthoeca*–specific primers was shown by amplification studies of genomic DNA of *A. phagocytophilum*, *Ehrlichia chaffeensis*, *E. canis*, *N. risticii*, *N. sennetsu*, and *N. helminthoeca*. All amplicons were separated by electrophoresis in 1% agarose gels and purified before cloning (pGEM-T and pGEM-T Easy Vector Systems, Promega, Madison, WI, USA) and sequencing. The Maringá sequences obtained were compared with those in GenBank by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic trees, sequence alignments, and identity tables were created by using Vector NTI Advance10 Software (Invitrogen, Carlsbad, CA, USA). GenBank accession numbers of *Anaplasmataceae* and their phylogenetic relationships are shown in the Figure.

Two dogs (N40–05, mesenteric lymph node, Maringá 1 and N20–04, Peyer's patch, Maringá 2) contained *Neorickettsia* spp. *rrs*, *rpo*B, or *gro*ESL genes. Both samples produced partial sequences for *Neorickettsia* spp. *rrs* gene; a similarity of 99% was observed for the 2 Maringá dog *rrs* sequences with *N. sennetsu*, *N. risticii*, and the *Stellantchasmus falcatus* (SF) agent. However, *N. helminthoeca rpo*B and *gro*ESL partial sequences were obtained only from dog 1. DNA identities of 100%, 82%, and 81% were observed between Maringá dog 1 sequences and *N. helminthoeca*, *N. risticii*, and *N. sennetsu* for the *rpo*B genes, respectively. All dogs were negative when tested with *glt*A gene primers. We observed 100% identity between the Maringá dog 1 sequence and *N. helminthoeca gro*ESL gene sequences. Similarities of 84%, 80%, and 79% were observed with *N. sennetsu*, the SF agent, and *N. risticii*, respectively. All positive controls showed bands of appropriate sizes, whereas negative controls yielded no products, confirming lack of amplicon contamination.

This study demonstrates that 2 dogs from Maringá, Brazil, with pathologic lesions consistent with SPD (*7*) were infected with a *Neorickettsia* sp. The partial sequences from dog 1 were identical to *N. helminthoeca rrs*, *gro*ESL, and *rpo*B genes, confirming infection with this organism (*2*). To our knowledge, this is the first confirmed description of this organism beyond the known geographic area of SPD. The organism identified in Brazil has been named *N. helminthoeca* Maringá strain.

Because of difficulty in recovering DNA from samples, need for a highly efficient PCR targeting small DNA regions, and limited sensitivity of the amplifications, sequences obtained

Figure. Neighbor-joining phylogenetic trees of the 16S rRNA (*rrs*), RNA polymerase β-subunit (*rpo*B), and heat-shock protein (*gro*ESL) gene sequences of *Anaplasmataceae* families. Trees were constructed with Vector NTI Advance10 Software (Invitrogen, Carlsbad, CA, USA). Bars represent substitutions per 1,000 bp. GenBank sequence accession numbers are provided.

for *N. helminthoeca* Maringá dog 1 (112 bp for *rrs*, 92 bp for *gro*ESL, 143 bp for *rpo*B) were short compared with those in GenBank (*rrs* 1,453 bp, *gro*ESL 1,914 bp, *rpo*B, 464 bp). Efficiency and sensitivity of targeting small DNA regions was necessary since storage and shipment of frozen samples were not optimal. Small DNA sequences are often suboptimal for delineation of phylogenetic relationships.

Bootstrapping analyses showed poor resolution (<380/1,000 iterations) below the genus level for the short *rrs* region examined. However, both the short *rpo*B and *gro*ESL regions examined had high bootstrap values (941/1,000 and 995/1,000 iterations, respectively). This finding allowed differentiation of *N. helminthoeca* and the Brazilian dog strain from *N. sennetsu*, *N. risticii*, and other related *Anaplasmataceae* and provided a high degree of confidence in the identification. More work is being implemented to obtain longer sequences to confirm and extend these genotypic comparisons. We propose further study to isolate the pathogen from other dogs for comparative biologic analyses.

Although SPD is caused by *N. helminthoeca*, infections by other *Neorickettsia* spp., including *N. risticii* (Potomac horse fever) and *N. sennetsu* (sennetsu fever), illustrate the potential of these widely distributed species to infect and cause disease in mammals and humans. Detection of *N. helminthoeca* in Brazilian dogs extends the range of this species and warrants a broad search for infections and spectrum of disease of *Neorickettsia* in animals and humans.

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References

1. Dumler JS, Rikihisa Y, Dasch GA. Family II *Anaplasmataceae*. In: Garrity GM, editor. Bergey's manual of systemic bacteriology. 2nd ed. Vol. 2. New York: Springer; 2005. p. 117–43.
2. Headley SA, Vidotto O, Scorpio D, Dumler JS, Mankowski J. Suspected cases of *Neorickettsia*-like organisms in Brazilian dogs. Ann N Y Acad Sci. 2004;1026:79–83.
3. Taillardat-Bisch AV, Raoult D, Drancourt M. RNA polymerase β-subunit-based phylogeny of *Ehrlichia* spp., *Anaplasma* spp., *Neorickettsia* spp. and *Wolbachia pipientis*. Int J Syst Evol Microbiol. 2003;53:455–8.
4. Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and "HGE agent" as subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001;51: 2145–65.
5. Rikihisa Y, Zhang C, Kanter M, Cheng Z, Ohashi N, Fukuda T. Analysis of p51, groESL, and the major antigen P51 in various species of *Neorickettsia*, an obligatory intracellular bacterium that infects trematodes and mammals. J Clin Microbiol. 2004;42:3823–6.
6. Inokuma H, Brouqui P, Drancourt M, Raoult D. Citrate synthase gene sequence: a new tool for phylogenetic analysis and identification of *Ehrlichia*. J Clin Microbiol. 2001;39:3031–9.
7. Cordy DR, Gorham JR. The pathology and etiology of salmon disease. Am J Pathol. 1950;26:617–37.

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Correction: Vol. 12, No. 4

In "Potential Arbovirus Emergence and Implications for the United Kingdom" by Ernest A. Gould et al., an error occurred on page 549. The first paragraph of the article incorrectly states that African horse sickness virus is circulating in Europe. The sentence should read "Finally, the family *Reoviridae* contains a variety of animal arbovirus pathogens, including bluetongue virus, which is currently circulating in Europe, and African horse sickness virus, which has been found in Europe but is not currently circulating."

The corrected text appears in the online article at http://www.cdc.gov/ncidod/EID/vol12no04/05-1010.htm

We regret any confusion this error may have caused.

Mycobacterium bovis Infection in Animals and Humans, 2nd Edition

Charles O. Thoen, James H. Steele, and Michael J. Gilsdorf, editors

Blackwell Publishers, Boston, Massachusetts, 2006
ISBN: 0813809193
Pages: 329; Price: US $124.99

The stated purpose of the second edition of *Mycobacterium bovis* Infection in Animals and Humans is to provide medical professionals, allied health scientists, research workers, and graduate students with current information on the significance of *M. bovis* in the control and eradication of tuberculosis in animals and humans. This newest edition deals with topics such as the public health significance of *M. bovis*, pathogenesis of *M. bovis*, epidemiology of *M. bovis* (with an entire chapter on molecular epidemiologic techniques), PCR detection of *M. bovis* with formalin-fixed tissues, and DNA vaccines. As with the first edition, the second edition delivers several updates from various countries on the status of *M. bovis* infection in animals and humans. Little accessible published information has been available on this topic, which makes the book especially useful.

The second edition also deals with several areas not covered in the first edition, including molecular epidemiology, evolution of the *M. tuberculosis* complex, tuberculosis caused by *M. pinnipedii* in fur seals and sea lions, the economics of bovine tuberculosis, and cost-benefit analysis of disease eradication programs. Several chapters deal with timely issues related to tuberculosis in wildlife.

In spite of its strengths, the second edition adds little additional information to material provided in the first edition on the topics of pathogenesis or diagnosis of bovine tuberculosis. In addition, although most of the photomicrographs are adequate, several are of such poor quality that they are of little use. Also, as with any multiauthored volume, some repetition occurs on general topics. The book achieves its stated purposes, however, and will be especially useful as a reference for researchers, regulatory agencies, and graduate students. It will be less informative for those interested in detailed discussions on research in the field of pathogenesis or diagnosis of *M. bovis* infection.

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Evolution of Microbial Pathogens

H. Steven Seifert and Victor J. DiRita, editors

American Society for Microbiology Press, Washington, DC, 2006
ISBN: 1-55581-300-3
Pages: 355; Price: US $119.95

This book is one of the first to provide an up-to-date view on a fundamental issue in medical microbiology research: how the accumulated genetic and genomic information is contributing to our understanding of virulence factors and the evolution of virulence in microbial pathogens. The editors should be commended for assembling 35 outstanding contributors, who specialize in various areas of microbial pathogenesis and evolution.

The 14 chapters are grouped into 3 broad sections: general concepts in microbial evolution, environment and the evolution of microbial pathogens, and the evolution of selected pathogenic species and mechanisms. At the beginning of each section, a concise overview of individual chapters integrates the content of the chapters into the section.

In the first section, the 5 chapters introduce the basic processes affecting microbial evolution, from the individual molecular level to the genomic, cellular, and population levels. Well-known concepts such as horizontal (lateral) gene transfer, the relationship between virulence and transmission, and pathogenicity islands are discussed extensively. Of special note are 2 chapters that are often missing in traditional medical microbiology books: 1 describes how long-term experimental evolutionary studies in the laboratory can contribute to our understanding of microbial pathogen evolution in the environment and clinics, and the other describes how gene inactivation and gene loss can be creative forces during the evolution of many microorganisms, especially obligate intracellular pathogens.

In the second section, the 5 chapters review how interactions between microbes and various natural biotic and abiotic factors can influence the origin and evolution of virulence in microbial pathogens. These factors are the physical, chemical, and biologic properties of the soil environment; the plant and animal environments; and to a lesser extent, the

aquatic and atmospheric environments. Other highly topical issues are the evolutions of toxins, secretion systems, and antibiotic resistance.

In the third section, the 4 chapters extensively discuss the evolution of selected groups of microbial pathogens: group A *Streptococcus* and *Staphylococcus aureus*; enteric pathogens such as *Escherichia coli*, *Salmonella enterica*, and *Yersinia* spp.; *Mycobacterium* spp.; and fungal pathogens such as *Candida albicans* and *Cryptococcus neoformans*. The authors provide rich detail of molecular variation within and between populations of these species and describe how patterns of population genetic variation have contributed to our understanding of the evolution of virulence and virulence factors in these pathogens.

I have no major criticism of what is included in this book; rather, I note what is absent, which could have made the book more comprehensive. The first is an overall evolutionary framework of the distribution of microbial pathogens on the phylogenetic tree. Such a macro-evolutionary framework would showcase the nonrandom patterns of the distribution of human pathogens among major phylogenetic groups of microorganisms. Second, although base substitutions, insertions and deletions, homologous recombination, and lateral gene transfer are discussed throughout the book, a generalized quantitative review of the relative contributions of these processes during the evolution of certain groups of microbial pathogens (e.g., *E. coli*) would have been highly informative. These processes are fundamental to the evolution of all groups of organisms, and the analysis of the unparalleled datasets in microbial pathogens can teach us much about the evolution of other groups of organisms. Third, although many human pathogens are globally distributed, a substantial number show geographic specificity and endemism. Therefore, the spatial and temporal patterns of distribution of microbial pathogens within a species and at the species level across the globe are highly relevant to the evolution of microbial pathogens. Lastly, this book is highly biased toward bacterial pathogens. Only 1 chapter deals with nonbacterial (fungal) pathogens, and no chapter discusses viral or protozoan pathogens, which are responsible for some of our biggest public health threats, e.g., HIV, influenza A, and *Plasmodium falciparum*.

Nevertheless, this is a timely and much-needed book about the evolution of bacterial virulence and its pathogenesis. It will be a valuable resource for researchers in the field of microbial evolution and pathogenesis, senior undergraduate students, graduate students, faculty who teach medical microbiology and microbial evolution, clinical microbiologists, and infectious disease specialists.

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Correction: Vol. 12, No. 6

In "Coccidioidomycosis as a Common Cause of Community-acquired Pneumonia," by Lisa Valdivia et al., an error occurred in the last sentence on page 961 that continues on page 962. The sentence should read "By using these entry criteria, we found that 3 of the 12 patients with valley fever who underwent radiographic examination had normal radiographs, which is consistent with results of a previous study (*3*), but did not adhere to Infectious Diseases Society of America or American Lung Association definitions of pneumonia (*19*)."

The corrected text appears in the online article at http://www.cdc.gov/ncidod/EID/vol12no06/06-0028.htm

We regret any confusion this error may have caused.

Corrections: Vol. 12, No. 7

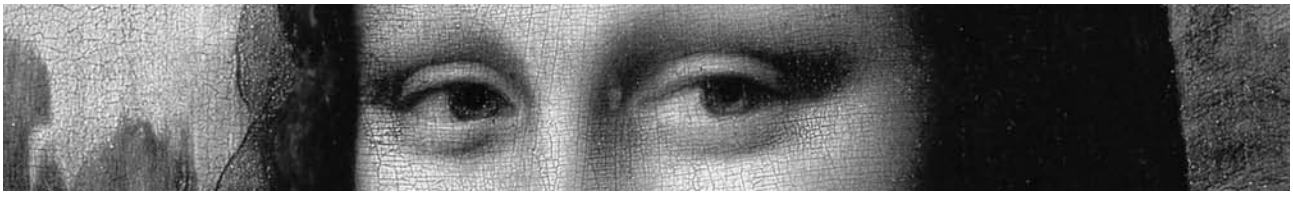
In "Migratory Passerine Birds as Reservoirs of Lyme Borreliosis in Europe," by Pär Comstedt et al., an error occurred in the second sentence of the first paragraph of Acknowledgments, page 1094. The sentence should read "This is report no. 214 from the Ottenby Bird Observatory."

The corrected text appears in the online article at http://www.cdc.gov/ncidod/EID/vol12no07/06-0127.htm

In "Human West Nile Virus Infection, Catalonia, Spain" by Domingo Bofill et al., an error occurred on page 1164. The final paragraph of the article incorrectly states that 20% of cases of West Nile virus infection are asymptomatic. The sentence should read "The probable WNV infection described was asymptomatic, as occurs in 80% of cases."

The corrected text appears in the online article at http://www.cdc.gov/ncidod/EID/vol12no07/06-0164.htm

We regret any confusion these errors may have caused.



Leonardo da Vinci (1452–1519). Mona Lisa (c.1503–1506). Oil on panel (77 cm × 53 cm). Copyright Louvre, Paris, France/Giraudon/ The Bridgeman Art Library Nationality/copyright status: Italian/out of copyright

Art, Science, and Life's Enigmas

Polyxeni Potter*

Vinci, a small town near Florence, Italy, dates back to Roman times, when it was inhabited by the Etruscans. "But this town is even more renowned for having given its name to the famous Leonardo da Vinci, who, in any discipline of science and art he dedicated himself to, surpassed all his contemporaries" wrote Emanuele Repetti in his geographic dictionary of Tuscany (*1*). In the modern sense, during his lifetime, the great Leonardo had no surname—"da Vinci" means "from Vinci."

Born out of wedlock to a notary-craftsman and a peasant woman, Leonardo was nonetheless well educated in Florence. At this cultural center, home of the Medici, he was apprenticed to sculptor and painter Andrea Del Verrocchio (*2*). "Marvelous and divine, indeed, was Lionardo the son of ser Piero da Vinci" said writer and painter Giorgio Vasari in his Lives of the Artists. During his apprenticeship, charged with painting an angel in Verrocchio's The Baptism of Christ, he painted a face so divine that Verrocchio never touched colors again "angry that a boy should know more than he" (*3*).

"He is a poor pupil who does not surpass his master," Leonardo noted, when his mathematical knowledge exceeded his tutor's (*4*). Botanist, architect, civil and military engineer, town planner, hydrologist, cartographer Leonardo anticipated, 500 years ago, the scientific discoveries of our time. "He made models of mills and presses, and machines to be worked by water, and designs for tunneling through mountains, and levers and cranes for raising great weights, so that it seemed that his brain never ceased inventing; and many of these drawings are still scattered about" (*3*). Though his radical notions (aviation, military hardware, mechanical calculation) escaped his contemporaries, his genius was widely acknowledged in his lifetime. Yet, much of his work has been lost to his flamboyance. Many projects were unfinished or obscured by secrecy and cryptic records. He wrote backwards with the left hand, so notes meant for him alone could be read only in the mirror. Only a dozen or so paintings survive.

At age 30, handsome and gifted, a musician who improvised verses on a lute of his own invention, Leonardo owned a studio in Milan and had several apprentices. He completed his first large painting, Virgin of the Rocks, and the masterpiece Last Supper. Then, he went to Venice and back to Florence to work as military engineer. He traveled to Mantua and Rome, where Raphael and Michelangelo worked, then to Pavia and Bologna. Finally, he left Italy for France and the court of humanist King Francis I. Though he painted little in France, he brought with him some of his great works, including Mona Lisa, which remained there after his death in the king's arms at age 67.

Art and science were aligned harmoniously in Leonardo. Art was guided by science, and science was expressed through art. His studies and experiments, begun in Verrocchio's workshop, mentioned in his correspondence with Ludovico, and recorded in copious notebooks, are masterfully illustrated. To paint better, he studied anatomy, dissecting human bodies and drawing them in detail. His work with optics, especially prisms, which anticipated Newton's, refined his rendition of light and shadow. His figures showing insertion of the muscles and their movements are still admired by anatomists. "It is true that decorum should be observed," he believed, "that is, movements should announce the motion of the mind of the one who is moving" (*4*).

"Lionardo was so pleased whenever he saw a strange head or beard or hair of unusual appearance that he would follow such a person a whole day, and so learn him by heart, that when he reached home he could draw him as if he were present" (*3*). Guided less by his extraordinary talent and more by meticulous technique, he shunned tradition and theory. In the most ubiquitous portrait of all time,

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Mona Lisa, which graces museums, dormitories, billboards, wine bottles, and now the cover of Emerging Infectious Diseases, the artist paid lip service to the formal vocabulary of Florentine tradition: a half-length figure, turned almost directly toward the viewer, beauty emanating from inner virtue.

Then, he positioned the figure up front to increase drama and intensity. Breaking with tradition, he painted a landscaped background, spatial depth. Instead of outlining the portrait, he merged it with surroundings. Perfecting sfumato, a technique described in antiquity by Pliny, he created an imperceptible transition between light and dark and sometimes between colors, "smoking" harsh edges with brushstrokes invisible to the naked eye. Cognizant of the way light fell on curved surfaces, he used layers of transparent color to capture it on gauzy veil or skin. The result was ethereal, magical, a glow that transformed portraiture for the ages, demanding not just likeness but the embodiment of spirit.

"As art may imitate nature, she does not appear to be painted, but truly of flesh and blood. On looking closely at the pit of her throat, one could swear that the pulses were beating," wrote Vasari (*3*). Mona Lisa, he continued, was painted for Florentine silk merchant Francesco del Giocondo, who commissioned it for his wife Lisa Gherandini, Mona (Madame) Lisa del Giocondo (La Gioconda), to mark the birth of their second son. Leonardo worked on the painting for several years and parted with it only at death.

"Executed in a manner well calculated to astonish all who behold her," the portrait was prominently displayed, admired, and widely reproduced (*3*). Raphael created a series of portraits with a striking resemblance, and among others, dadaist Marcel Duchamp and surrealist Salvador Dalí produced their mock interpretations. Yet, "those who put the moustache on Mona Lisa," wrote contemporary artist Barnett Newman, "are not attacking it or art, but Leonardo da Vinci the man. What irritates them is that this man with half a dozen pictures has this great name in history, whereas, they, with their large oeuvre, aren't sure" (*4*).

"Mona Lisa being most beautiful, he used while he was painting her, to have men to sing and play to her and buffoons to amuse her, to take away that look of melancholy which is so often seen in portraits; and in this of Lionardo's there is a peaceful smile more divine than human" (*3*). Much has been speculated about the smile, about the painting, about Leonardo. While Vasari was acquainted with the Giocondo family, he did not write his anecdotal biography until more than 30 years after Leonardo's death, and competing accounts of Mona Lisa's origin and identity abound. If the portrait was commissioned, why was it never delivered to its patron? Leonardo himself left scant evidence of his own opinions and ideas and only one definitive self-portrait in red chalk, a venerable face carved by time, framed by flowing hair and beard.

Some attribute the uncanny perfection of Mona Lisa to Leonardo's scientific observation, mathematical instinct, unparalleled skill, and the harmony of the composition (*5*). Others take different paths: "The elusive quality of Mona Lisa's smile…is almost entirely in low spatial frequencies, and so is seen best by your peripheral vision" (6).

The subtle smile, reminiscent of archaic funerary statues (kouroi, korai), the languid eyes, the puzzling backdrop of nature, the intricate loops of the neckline, the calm hands, even the absence of visible facial hair (eyebrows and eyelashes were not the style) add to the mysterious, semi-abstract quality of the face.

The enigma of Leonardo's creation and the intrigue surrounding its origin, identity, and meaning can only be a metaphor for his own life and ideas and, by extension, ours. An archetype of the Renaissance, this man who would and could do everything must have peered inside himself for answers he had sought far afield. And just as he dissected and outlined the physical body, he sought to find and paint the spirit. Always the scientist, he knew that a portrait alone, no matter how exacting, would not do. Rejecting the flat background of tradition, he added the landscape. Part dream, part romantic reality, it provided perspective and connected the figure to the world, adding to the enigma and possibly holding the definitive interpretation.

The puzzles of our era, unknown pathogens, many of them vectorborne, emerging biological threats, ecologic disasters, antimicrobial drug resistance can also benefit from meticulous observation, accurate recording, added perspective, and the interdisciplinary approach to knowledge. Just as with Leonardo, the art is in the science.

References

1. Repetti, E. *Dizionario geografico fisico storico della Toscana contenente la descrizione di tutti i luoghi del granducato ducato di Lucca, Garfagnana e Lunigiana*. 5 vols. and appendix. Florence; 1833–1846.
2. Leonardo da Vinci. [cited 2006 Jun]. Available from http://en.wikipedia.org/wiki/Leonardo_da_Vinci
3. Giorgio Vasari's lives of the artists. [cited 2006 Jun]. Available from http://www.fordham.edu/halsall/basis/vasari/vasari14.htm
4. Zöllner F, Nathan J. Leonardo da Vinci: the complete paintings and drawings. London: Taschen; 2003.
5. Mona Lisa. [cited 2006 Jul]. Available from http://www.pbs.org/treasuresoftheworld/mona_lisa/mlevel_1midentity.html
6. Mona Lisa smile secrets revealed. [cited 2006 Jun]. Available from http://newsvote.bbc.co.uk/mpapps/pagetools/print/news.bbc.co.ukl/2/hi/entertainment/2775

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the September issue for the following topics:

Risk Factors for Buruli Ulcer, Benin

Nosocomial Tuberculosis Transmission, India

Quantitative Histology and Immunodetection of African Tick-bite Fever

Controlling Avian Influenza in Poultry

Mycobacterium tuberculosis Ancestral Lineages in India

Lymph Node Biopsy and Cat-scratch Disease

Extrapulmonary Tuberculosis in the Netherlands, 1993–2001

Risk for Tuberculosis in Children

Multidrug-resistant Tuberculosis Treatment in Resource-limited Settings

Fluoroquinolones as Risk Factors for Methicillin-resistant *Staphylococcus aureus* in Quebec

Differentiation of Tuberculosis Strains among Beijing-family Strains

Periurban *Trypanosoma cruzi*–infected *Triatoma infestans*, Arequipa, Peru

Distinguishable Genomic Signatures of Human and Avian Influenza A Viruses

Complete list of articles in the September issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 3, 2006
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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

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